TITLE OF INVENTION: "RECEPTOR-LIGAND SYSTEM AND ASSAY"

INVENTORS:

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DESCRIPTION

BACKGROUND OF THE INVENTION

Technical Field

THIS INVENTION relates to the Eph family of receptor tyrosine kinases, to the high-affinity ligand-binding site of such receptors, and to methods whereby Eph receptor agonists and antagonists may be identified. In particular, the invention relates to the Eph family receptor HEK. Because of the highly conserved nature of the receptor tyrosine kinases of the Eph family, the methods of the invention are applicable to other members of this family such as EPH, ECK and ERK. Generally, Eph receptor tyrosine kinases are involved in embryonic development of the brain and nervous system, leukaemias and solid tumors, and may have a role in metastasis.

Background

Increasing interest in understanding the molecular basis of tissue modeling and patterning processes in vertebrate development has led to the identification of protein families which direct cell movement in embryogenesis (reviewed by Bonhoeffer & Sanes, 1995, Curr. Opin. Neurobiol. **5** 1-5). Apart from members of the fibroblast growth factor (FGF) and transforming growth factor beta (TGF-β) families, which are involved in mesoderm induction and patterning (Green & Smith, 1991, Trends in Genetics **7** 245-250), proteins of the netrin, semaphorin and collapsin families are thought to control axon guidance and neural

pathfinding (Kennedy & Tessier-Lavigne, 1995, Current Opinion in Neurobiology **5** 83-90; Müller *et al.*, 1996, Current Opinion in Genetics and Development **6** 469-474).

Such growth factors and their cell surface receptors, as well as many other types of receptor-ligand pairs, have characteristic mechanisms for transducing the ligand-receptor binding effect into intracellular changes.

One major receptor type is the receptor protein-tyrosine kinase (RTK) family, the members of which include intracellular tyrosine kinase domains which are activated in response to ligand stimulation, resulting in autophosphorylation of certain receptor tyrosine residues. The phosphorylated tyrosines in turn bind to and activate signaling molecules, thereby activating an intracellular signaling cascade.

Over 14 distinct groups of RTKs are known, and of these the largest group is the "Eph" family, which until comparatively recently were "orphan" receptors for which no ligand had been identified. However, the Eph family ligands are now known to represent a family of glycosyl phosphatidylinositol (GPI)-linked or transmembrane molecules.

Among RTKs which are implicated in the regulation of developmental patterning events (Pawson and Bernstein, 1990, Trends Genet. **6** 350-356), members of the Eph-family RTKs have been linked to neurogenesis (Müller *et al.*, 1996, *supra*; Tessier-Lavigne, M., 1995 Cell **82** 345-348; Pandey *et al.*, 1995 **5** 986-989; Nieto, M.A., 1996, **17** 1039-1048) initially due to their spatially-restricted expression patterns during the development of the vertebrate nervous system (reviewed by Friedman & O'Leary, 1996, Current Opinion in Neurobiology **6** 127-133). The characterisation of the expression patterns together with functional studies of Eph receptors has, in several cases, confirmed significant roles for Eph signaling in axon guidance, in particular, during the development of the retinotectal projection map (Cheng & Flanagan, 1994, Cell **79** 157-168; Cheng *et al.*, 1995, Cell **82** 371-381; Drescher, U., 1995, Cell **82** 359-370; Winslow, *et al.*, 1995, Neuron **14** 973-981; Tessier-Lavigne, 1995, *supra*;

Brennan *et al.*, 1997, Development **124** 655-664).

The results of studies utilizing overexpression of some family members including HEK, EPH, ERK and ECK in tumour-derived cell lines, tumour specimens and transfected cells implicates these receptors in oncogenesis (Hirai *et al.*, 1989, Sciene **238** 1717-20; Boyd *et al.*, 1992, J. Biol. Chem. **267(5)** 3262-7; Maru *et al.*, 1990; Andres *et al.*, 1994, Oncogene **9** 1461-7).

HEK was first identified on the cell surface of a pre-B acute lymphoblastic leukemia cell line, LK63, using the III-A4 monoclonal antibody (Boyd *et al.*, 1992, *supra*). Immunofluorescence studies with III-A4 revealed expression of HEK in blood samples from patients with acute leukemia, but not in normal adult tissues or blood cells (Boyd *et al.*, 1992, *supra*; Wicks *et al.*, 1992, Proc. Natl. Acad. Sci. (USA) **89(5)** 1611-5).

A cDNA encoding HEK has been obtained and the nucleotide sequence of the entire coding region deduced as reported in Wicks *et al.*, 1992, Proc. Natl. Acad. Sci USA (which is herein incorporated by reference), and in WO93/00425 (which is herein incorporated by reference).

In embryos, the expression patterns of the murine and chicken HEK homologues MEK 4 and CEK 4, and their recently identified respective ligands ELF1 and RAGS, suggest a role in the development of the retinotectal projection map. A soluble HEK ligand from human placenta conditioned medium has been identified using a biosensor-based affinity detection approach (Lackmann *et al.*, 1995). The HEK ligand was identified by sequence homology as a soluble form of AL-1 (Winslow *et al.*, 1995, Neuron **14** 973-981), a member of the family of ligands for EPH Related Kinases (LERKS; Bohme *et al.*, 1996, J. Biol. Chem. **271** 24727-24752; Cerreti *et al.*, 1996, Genomics **35** 376-379), which for consistency with other members will hereinafter be referred to as LERK 7. This family of transmembrane or membrane-associated proteins were isolated as potential ligands for EPH-like RTKs through their interactions with recombinant EPH receptor family exodomains (Winslow *et al.*, 1994;

Beckmann *et al.*, 1994, Embo Journal. **13** 3757-62; Shao *et al.*, 1995, Journal of Biological Chemistry **270** 3467-70; Brambilla *et al.*, 1995, Embo Journal **14** 3116-3126).

Extremely high interspecies sequence similarities of the known Eph family members suggests that these receptors have evolutionarily conserved functions, but little is known about the actual protein structures or about the structure/function relationships between Eph-like receptors and their ligands. Typically, and as is the case with HEK, Eph RTKs have an exodomain which includes an N-terminal cysteine-rich region, the outer portion of which has been described as immunoglobulin-like (Ig-like), and two fibronectin III regions (Pandey *et al.*, 1995, Journal of Biological Chemistry **270** 19201-19204; Tuzi & Gullick, 1994, British Journal of Cancer **69** 417-421; Henkemeyer, M., 1994, Oncogene **9** 1001-1014). Extensive cross-reactivity of Eph receptor/ ligand interactions has been observed with divalent receptor (ligand) fusion proteins containing the Fc domain of human IgG 1 (Beckmann *et al.*, 1994, supra; Davis *et al.*, 1994, Science **266** 816-819; Pandey *et al.*, 1994, Journal of Biological Chemistry **269** 30154-30157; Cerretti *et al.*, 1995, Molecular Immunology **32** 1197-1205; Pandey *et al.*, 1995, Current Biology **5** 986-989; Brambilla *et al.*, 1995, *supra*).

All of the known ligands exist as membrane-associated forms, and dependence of receptor activation on membrane bound or oligomerised ligands (Winslow *et al.*, 1995, *supra*; Davis *et al.*, 1994, *supra*) was reported for most members of the Eph-like receptor and ligand families. The apparent receptor/ligand promiscuity of various receptors and ligands monitored with receptor or ligand Fc fusion constructs suggested that Eph family RTKs could be separated into two redundant sub-classes, based on affinity for transmembrane of GPI-linked respectively. Together with their overlapping expression patterns, this led to the formulation of a model in which promiscuous interactions within subclasses mediates formation of spatial boundaries and patterning events during development (Gale *et al.*, 1996, Neuron **17** 9-19).

This reported redundancy is at odds with several studies which demonstrate specialised functions of the homologous RTKs MEK4/CEK4/RTK2 and their corresponding ligands ELFI/RAGS/zEphL4 during the development of the retinotectal projection map in mouse, chicken and zebrafish (Cheng *et al.*, 1995, Cell **82** 371-381; Drescher *et al.*, 1995, Cell **82** 359-370; Nakamoto *et al.*, 1996, Cell **86** 755-766; Brennan *et al.*, 1997, Development **124** 655-664).

OBJECT OF THE INVENTION

The present inventors have realized that in order to determine the specific function of each Eph RTK, it is essential that the nature of the ligand-receptor interaction relevant to each Eph family RTK be resolved.

With this realization in mind, the present inventors have identified LERK7 as the preferred high-affinity ligand for HEK, and thereby located a domain within HEK which is responsible for binding the high-affinity ligand. The previously-mentioned high level of sequence similarity between EpH-family RTKs suggests that similarly located ligand-binding domains exist in all Eph family RTKs.

It is therefore an object of the invention to provide an Eph family RTK ligand-binding domain.

It is a further object of the invention to provide a method of identifying Eph family RTK agonists or antagonists.

DISCLOSURE OF INVENTION

In one aspect, the invention provides a ligand-binding domain of a receptor protein kinase (RTK) of the Eph family.

Preferably, the Eph-family RTK is HEK.

Preferably, the ligand which binds the ligand-binding domain is LERK7.

Preferably, the ligand-binding domain comprises at least one disulphide bond involving cysteine residues corresponding to conserved cysteine residues in HEK which are selected from the group consisting of:

(i) CYS_{71}-CYS_{189};

(ii) CYS_{257}-CYS_{270};

(iii) CYS_{259}-CYS_{270};

(iv) CYS_{306}-CYS_{322}; and

(v) CYS_{362}-CYS_{365}.

Preferably, the ligand-binding domain is encoded by exon III of a gene encoding said RTK of the Eph family.

Preferably, the ligand-binding domain additionally includes an amino acid sequence encoded by exon II of said gene.

Most preferably, the ligand-binding domain includes an amino acid sequence encoded by exon I, exon II and exon III of said gene.

Furthermore, and as will be discussed hereinafter, a ligand-binding domain comprising an amino acid sequence encoded by exon I, exon II and exon III of said gene is particularly useful for recombinant expression.

Preferably, said ligand-binding domain is a polypeptide having amino acids 52-271 of the sequence shown in FIG. 1 (SEQ ID NO:1).

Preferably, said ligand-binding domain polypeptide further includes one or more of amino acids 30-51 of the sequence shown in FIG. 1 (SEQ ID NO:2).

Preferably, said ligand-binding domain polypeptide further includes one or more of amino acid residues 1-29 of the sequence shown in FIG. 1 (SEQ ID NO:3).

As previously mentioned, a ligand-binding domain of an Eph-family RTK which is particularly useful for recombinant expression consists of amino acids 1-271 of the sequence shown in FIG. 1 (SEQ ID NO:4).

It will be appreciated that the amino acid sequence shown in FIG. 1 (SEQ ID NO:4) corresponds to that encoded by exons I, II and III of the human HEK gene.

In this regard, and as will be described hereinafter, exon III encodes amino acids which appear to be necessary for ligand binding by Eph family RTKs. However, it is also clear that amino acid sequences flanking the exon III-encoded sequence may also form part of the ligand-

binding domain, perhaps by contributing to correct folding or other structural requirements of the ligand-binding domain. The functional importance of the exon III-encoded amino acid sequence will also be demonstrated hereinafter.

In a second aspect, the invention provides a polynucleotide sequence as shown in FIG. I (SEQ ID NO:5), wherein:-

(i) nucleotides 1-87 correspond to exon I of the HEK gene (SEQ ID NO:6);

(ii) nucleotides 88-153 correspond to exon II of the HEK gene (SEQ ID NO:7); and

(iii) nucleotides 154-813 correspond to exon III of the HEK gene (SEQ ID NO:8).

The present invention also provides homologs of the polynucleotide sequence of the invention, which homologs include:-

(1) all polynucleotide sequences encoding polypeptides of the invention; and

(2) all polynucleotide sequences encoding subsequences of polypeptides of the invention.

As used herein, "sub-sequences of polypeptides of the invention" are polypeptides of the invention which have one or more amino acid sequence deletions, but which retain the functional characteristics of the polypeptide of the invention.

In this regard, a skilled addressee would also realize that advantage can be taken of codon sequence redundancy so as to incorporate changes in a nucleotide sequence without affecting the encoded amino acid sequence.

Furthermore, a skilled addressee would be aware that one or more nucleotides of the polynucleotide sequences of the invention could be substituted so as to produce one or more conservative amino acid changes that do not alter the functional characteristics of the polypeptides of the invention.

Thus, the homologs of the invention include altered polynucleotide sequences which encode polypeptides with the same functional characteristics as the polypeptides of the invention

The polynucleotide sequence homologs of the invention further comprise polynucleotide sequences that hybridize with polynucleotide sequences of the invention under substantially stringent conditions. Suitable hybridization conditions will be discussed hereinafter.

"Hybridization" is used here to denote the pairing of complementary bases of distinct polynucleotide sequences to produce a DNA-DNA hybrid, a DNA-RNA hybrid, or an RNA-RNA hybrid according to base-pairing rules.

In DNA, complementary bases are:-

(i) A and T; and

(ii) C and G.

In RNA, complementary bases are:-

(i) A and U; and

(ii) C and G.

In DNA-RNA hybrids, complementary bases are:-

(i) A and T;

(ii) A and U; and

(iii) C and G.

Typically, substantially complementary polynucleotide sequences are identified by blotting techniques that include a step whereby polynucleotides are immobilized on a matrix (preferably a synthetic membrane such as nitrocellulose), a hybridization step, a washing step and a detection step.

Southern blotting is used to identify a complementary DNA sequence; Northern blotting is used to identify a complementary RNA sequence. Dot blotting and slot blotting can be used to identify complementary DNA/DNA, DNA/RNA or RNA/RNA polynucleotide sequences. Such techniques are well known by those skilled in the art,

and have been described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Eds. Ausubel *et al.*, John Wiley & Sons Inc 1995) at pages 2.9.1 through 2.9.20. According to such methods, Southern blotting involves separating DNA molecules according to size by gel electrophoresis, transferring the size-separated DNA to a synthetic membrane, and hybridizing the membrane bound DNA to a complementary polynucleotide sequence labeled radioactively, enzymatically or fluorochromatically. In dot blotting and slot blotting, DNA samples are directly applied to a synthetic membrane prior to hybridization as above.

An alternative blotting step is used when identifying complementary polynucleotide sequences in a cDNA or genomic DNA library, such as through the process of plaque or colony hybridization. A typical example of this procedure is described in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL 2nd Ed (Cold Spring Harbour Press 1989) Chapters 8-12, which is herein incorporated by reference.

A skilled addressee will recognize that a number of factors influence hybridization, and that these factors can be manipulated to optimize the specificity of the hybridization.

Maximum hybridization typically occurs at about 20° to 25° below the T_m for formation of a DNA-DNA hybrid. Maximum hybridization typically occurs at about 10° to 15° below the T_m for a DNA-RNA hybrid.

It is well known in the art that the T_m is the melting temperature, or temperature at which two complementary polynucleotide sequences dissociate. Methods for estimating T_m are well known in the art (see CURRENT PROTOCOLS IN MOLECULAR BIOLOGY *supra* at page 2.10.8).

A commonly used empirical formula for calculating DNA T_m is:-

$$T_m = 81.5°C+16.6(\log M)+0.41(\%G+C) -0.61(\%\text{formamide})$$

-500/L

where M= molarity of monovalent cations

and L = sequence length

The specific activity of radioactively labeled polynucleotide sequence should typically be at least 10^8 dpm/μg to provide a detectable signal. A polynucleotide sequence radiolabeled to a specific activity in the order of 10^9 dpm/μg can detect approximately 0.5 pg of DNA. It is well known in the art that sufficient DNA must be immobilized on the membrane to permit detection. It is desirable to have excess immobilized DNA, usually 10 :g. Adding an inert polymer such as 10% (w/v) dextran sulfate (MW 500,000) or polyethylene glycol 6000 during hybridization can also increase the sensitivity of hybridization (see Ausubel *et al.*, *supra* at 2.10.10).

To achieve meaningful results from hybridization between a polynucleotide sequence immobilized on a membrane and a labeled polynucleotide sequence, a sufficient amount of the labeled polynucleotide sequence must be hybridized to the immobilized polynucleotide sequence following washing. Washing ensures that the labeled polynucleotide sequence is hybridized only to the immobilized polynucleotide sequences with a desired degree of complementarity to the labeled polynucleotide sequence.

"Stringency" as used herein, refers to the temperature and ionic strength conditions, and presence or absence of certain organic solvents, during hybridization. The higher the stringency, the higher will be the degree of complementarity between the immobilized polynucleotide sequences and the labeled polynucleotide sequence.

"Stringent conditions" designates those conditions under which only polynucleotide sequences having a high frequency of complementary bases will hybridize, and remain hybridized during washing.

For a detailed example of stringent conditions, see

CURRENT PROTOCOLS IN MOLECULAR BIOLOGY *supra* at pages 2.10.1 to 2.10.16, and Sambrook et al in MOLECULAR CLONING. A LABORATORY MANUAL (Cold Spring Harbour Press, 1989) at sections 1.101 to 1.104, which are hereby incorporated by reference.

Methods for detecting labeled polynucleotides hybridized to an immobilized polynucleotide are well known to practitioners in the art. Such methods include autoradiography, chemiluminescent, fluorescent and colorimetric detection.

It is also contemplated that polynucleotide sequence homologs may be obtained using polynucleotide sequence amplification techniques.

In this regard, the polynucleotide sequence homologs of the invention may be prepared according to the following procedure:-

(i) designing primers based on sub-sequences of a polynucleotide sequence of the invention; and

(ii) using said primers to amplify, via polynucleotide sequence amplification techniques, one or more fragments from a polynucleotide extract.

By "fragment" is meant a DNA product generated by polynucleotide sequence amplification techniques.

As used herein, "sub-sequences of polynucleotide sequences" are sequences of nucleotides contained within the polynucleotide sequence of the invention.

In this regard, it will also be appreciated that said primers may be degenerate, in which case nucleotide sequences thereof will be determined according to an amino acid sequence encoded thereby. That is, advantage may be taken of codon sequence redundancy to design said degenerate primers so that polynucleotide sequence homologs which have non-conserved nucleotide sequence(s) may be amplified according to this method.

Suitable polynucleotide sequence amplification techniques

are well known to the skilled addressee, and include polymerase chain reaction (PCR), strand displacement amplification (SDA) and rolling circle replication (RCR).

The polynucleotide extract may be in the form of a cDNA or genomic library. In this regard, the cDNA or genomic library may be derived from a eukaryote, including mammals such as humans or mice. Such libraries may comprise genomic DNA or cDNA ligated into vectors such as λ FIX II or λ DASH II, as will be described hereinafter.

Alternatively, said polynucleotide extract could be an mRNA extract obtained from cells or tissues which has been reverse transcribed to cDNA.

The polypeptides of the invention also include within their scope homologs, and sub-sequences thereof as previously defined.

A polypeptide homolog is a polypeptide of the invention with an altered amino acid sequence, such as through conservative amino acid substitution(s), but with unaltered functional characteristics.

A recombinant polypeptide of the invention may be prepared by a procedure comprising the steps of:-

(i) ligating a polynucleotide sequence of the invention into a suitable expression vector to form an expression construct;

(ii) transfecting or transforming a suitable host cell with said expression construct;

(iii) expressing said polypeptide of the invention; and

(iv) isolating said polypeptide of the invention.

It will be understood that this procedure is applicable to polypeptide sequences of the invention, homologs and sub-sequences thereof.

The expression construct comprises an expression vector, as is well known in the art, and a polynucleotide sequence of the invention which encodes a polypeptide of the invention, wherein the polynucleotide

sequence of the invention is operably linked to one or more regulatory nucleotide sequences present in the expression vector (such as a promoter, terminator and polyadenylation sequence) that will induce expression of the polypeptide of the invention.

Both constitutive and inducible promoters may be useful adjuncts for expression of polypeptides according to the invention. An expression vector according to the invention may be a plasmid cloning vector suitable for either prokaryotic or eukaryotic expression. Such vectors are well known to those skilled in the art.

A preferred eukaryotic expression vector which provides consitutive expression is pEFBOS, as will be described hereinafter.

In light of the foregoing, it will also be realized by those skilled in the art that the expression vector and the host cell used will be interdependent.

Preferred host cells for eukaryotic expression are Chinese Hamster Ovary (CHO) cells and COS 7 cells, as will be described hereinafter.

An expression construct may also include a fusion partner sequence (usually provided by the expression vector) so that the recombinant polypeptide of the invention is expressed as a fusion polypeptide with said fusion partner. The main advantage of fusion partners is that they assist identification and/or purification of said fusion polypeptide.

In order to express said fusion polypeptide, it is necessary to ligate the polynucleotide sequence of the invention into the expression vector so that the translational reading frames of the fusion partner and the polynucleotide sequence of the invention coincide.

Well known examples of fusion partners are glutathione-S-transferase (GST), Fc portion of human IgG_1, maltose binding protein (MBP) and hexahistidine (HIS_6), which are particularly useful for isolation of the fusion polypeptide of the invention by affinity chromatography. For

the purposes of fusion polypeptide purification by affinity chromatography, relevant matrices for affinity chromatography are glutathione-, amylose-, and nickel- or cobalt-conjugated resins respectively.

Another fusion partner well known in the art is green fluorescent protein (GFP). This fusion partner serves as a fluorescent "tag" which allows the fusion polypeptide of the invention to be identified by fluorescence microscopy or by flow cytometry. The GFP tag is useful when assessing subcellular localization of the fusion polypeptide of the invention, or for isolating cells which express the fusion polypeptide of the invention. Flow cytometric methods such as fluorescence activated cell sorting (FACS) are particularly useful in this latter application.

Preferably, the fusion partners also have protease cleavage sites, such as for Factor X_a or Thrombin, which allow the relevant protease to partially digest the fusion polypeptide of the invention and thereby liberate the recombinant polypeptide of the invention therefrom. The liberated polypeptide can then be isolated from the fusion partner by subsequent chromotagraphic separation.

Fusion partners according to the invention also include within their scope "epitope tags", which are usually short peptide sequences for which a specific antibody is available. Well known examples of epitope tags for which specific monoclonal antibodies are readily available include c-myc, influenza virus haemagglutinin and FLAG tags.

Preferably, the FLAG tag is used as an epitope tag with polypeptides of the invention. This epitope tag is preferably recognized by the anti-FLAG M2 monoclonal antibody (mAb). As will be demonstrated hereinafter, the presence of a FLAG-epitope does not appreciably alter the biological interaction between HEK and its high-affinity ligand LERK 7.

It will also be appreciated that polypeptides of the invention, homologs and sub-sequences thereof may be prepared by chemical synthesis, rather than by recombinant DNA methods.

Applicable methods of chemical synthesis are well known in the art, and it is customary for such methods to be automated and readily available to the skilled person. This approach is particularly relevant to the preparation of peptides, for example corresponding to subsequences of polypeptides of the invention. Suitable polypeptide synthesis procedures are described in detail in Chapter 18 of CURRENT PROTOCOLS IN PROTEIN SCIENCE, Coligan *et al.* Eds (John Wiley & Sons). Also, chemical synthesis of a peptide consisting of amino acid residues 1-31 of HEK will be described hereinafter.

In a third aspect, the invention provides a method of identifying a molecule which binds an Eph RTK, which method includes the steps of:-

(i) combining a sample suspected of containing said molecule and at least a ligand-binding domain of an Eph family RTK; and

(ii) determining if the molecule is present in the sample by measuring binding of said molecule to the ligand-binding domain.

The ligand-binding domain may have additional amino acid sequences or polypeptide domains. For example, it may be advantageous for said at least a ligand binding domain to have amino acids which are involved in dimerization of said Eph family RTK.

As used herein "sample" refers to any material which may potentially contain said molecule. It will be appreciated that said molecule could be a high-afinity ligand such as LERK7, in which case the method according to this aspect of the invention is useful for diagnostically detecting the ligand in samples such as body fluids, cell extracts, serum and the like.

Alternatively, this method is useful for identifying hitherto unknown Eph family RTK-binding molecules which mimic ligands such as LERK7. Such molecular mimics are hereinafter referred to as "agonists".

In a fourth aspect, the invention provides a method of identifying a molecule which competes with binding of a ligand to at least a ligand-binding domain of an Eph family RTK, which method includes the steps of: -

(i) combining a sample suspected of containing the molecule, a ligand and at least a ligand-binding domain of an Eph family RTK; and

(ii) determining if the molecule is present in the sample by measuring whether the molecule competes with said ligand for binding to said ligand-binding domain.

It will be appreciated that the method according to the third and fourth aspects of the invention may be suitable for identifying molecules which interfere with binding between Eph family RTKs and their ligands. Such molecules are hereinafter referred to as "antagonists".

It will be appreciated that there are numerous binding assays available to the skilled addressee which are suitable according to the methods of the third and fourth aspects.

Such assays include radioligand binding assays, affinity chromatography-based assays, equilibrium sedimentation analysis, and sensor chip-based assays such as using the BIAcore system.

Preferably, detection of agonists and/or antagonists is performed using assays wherein either the Eph family RTK ligand-binding domain or said ligand is immobilized on a solid support such as a microtitre plate-well, bead (e.g. CNBR-activated sepharose) or a sensor chip such as is used with the BIACore system.

Advantageously, the method according to the third and fourth aspects of the invention utilizes said BIACore system. The BIACore system provides an extremely sensitive and efficient technique which is well known to the skilled person. The operation of the BAICore system will be described in detail hereinafter.

In a fifth aspect, the present invention provides agonists

and/or antagonists of Eph family RTKs.

In this regard, it will be appreciated that the ligand-binding domain of the present invention may itself be an antagonist by virtue of its ability to compete with Eph family RTKs for ligand binding.

According to this aspect, it is preferable that the ligand is LERK 7 and that said Eph family RTK is HEK.

In a sixth aspect, the invention provides a method of determining whether or not an mRNA encodes an intact Eph family RTK ligand-binding domain, which method comprises the step of introducing the mRNA into a zebrafish embryo at the one-cell, two-cell, or four-cell stage, and detecting defects, if present, in early embryogenesis in the zebrafish embryo, said defects being indicative of said mRNA encoding said intact ligand-binding domain.

Preferably the mRNA has a nucleotide sequence corresponding to the first seven exons of an Eph family RTK gene.

Preferably, the Eph family RTK is HEK.

In a seventh aspect, the invention provides a method of determining whether or not an mRNA encodes an intact ligand for an Eph family RTK ligand-binding domain, which method comprises the step of transcribing the gene to mRNA, introducing the mRNA into a zebrafish embryo at the one-cell, two-cell, or four-cell stage, and detecting defects, if present, in early embryogenesis in the zebrafish embryo, said defects being indicative of said mRNA encoding said intact ligand.

Preferably, the ligand is LERK 7.

According to the sixth and seventh aspects, it is preferred that mRNA is introduced into the embryo by microinjection into the yolk cell immediately under the blastoderm. Suitable methods for preparing mRNA will be described hereinafter.

Preferably a syndrome comprising defects involving reduced dorsal axis height from the yolk cell, disorganised anterior neuraxis, and disorganised somite boundaries is detected.

More preferably this syndrome of defects is detected in at least 50% of embryos subjected to the test.

As will be discussed in more detail hereinafter, injection of mRNA encoding LERK 7 causes defects identical to the defects caused by soluble HEK comprising the ligand-binding domain. Furthermore, co-injection of receptor and ligand mRNA achieves a partial rescue of the phenotype, demonstrating the specificity of the receptor-ligand mediated effects.

In an eigth aspect, the invention provides a method of identifying the site of functional effects of interaction between an Eph family RTK ligand-binding domain and a ligand, comprising the steps of injecting zebrafish embryos with mRNA encoding the ligand-binding domain, and subjecting the embryos to *in situ* hybridisation with probes to *Hlx-1*, *Paxb*, *Krox20* and/or *MyoD*, and detecting patterns of *in situ* hybridisation consistent with aberrant gene expression.

Preferably the Eph family RTK is HEK, and the ligand is LERK 7. Preferably the method according to the eighth aspect is used to identify events occuring during embryogenesis.

The method according to the eighth aspect of the invention may also be useful for identifying Eph family RTK agonists and antagonists.

Eph family RTKs are associated with solid tumours such as melanoma and cancers of the colon, liver, lung, breast and prostate. Eph family RTKs play a role in metastasis possibly due their role in cell migration and tissue structure. In particular, HEK is associated with pre-B cell leukaemia and with other leukaemias. It is therefore considered that agonists and antagonists of the invention, such as LERK 7 agonists/antagonists, may be potentially useful as anti-cancer or anti-metastatic agents.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: Nucleotide sequence of exon I (nucleotides 1-87), exon II

(nucleotides 88-153) and exon III (nucleotides 154-813) of the HEK gene, and the deduced amino acid sequence. The nucleotide sequence of FIG. 1 was constructed using the data in Table 1 together with the HEK cDNA sequence as shown in Wicks *et al.*, 1992 *supra* and WO 93/00425 *supra*.

FIG. 2A: Correspondence between exon structure and subregions of the extracellular portion of Eph family RTKs.

FIG. 2B. Exon structure corresponding to various soluble HEK (sHEK) constructs. The exons are indicated by differential shading from left to right as follows: exons I+II, exon III, exon IV, exon V, exons VI + VII. The isolation of exons belonging to HEK, SEK1, BSK and ESK genes is described herein. Exon VII from the MEK4 gene was reported in Sajjadi *et al.* (1991).

FIG. 2C: Expression of soluble HEK (sHEK) polypeptides encoded by the constructs shown in B. sHEK in lane 1 corresponds to the complete extracellular domain (exodomain) of HEK encoded by exons I-VII. Soluble HEK I-VII in lane 2 includes a FLAG tag, hence the slightly slower migration.

FIG. 2D: sHEK proteins at increasing concentrations (15.6 - 500 nM) were injected onto LERK 7-derivatised sensor chips and the association rate constants, (M) derived from BIAcore raw data using the BIA evaluation software as described in *Materials and Methods*.

FIG. 2E: The kinetic rate constants were then used to estimate apparent dissociation constants according to K_D = kd/ka. The mean and standard deviation from estimates at five different concentrations are shown (dark shading). In addition, equilibrium responses were used to estimate the apparent equilibrium dissociation constants (light shading).

FIG. 2F: Samples containing 40 nM LERK 7 and 1 nM-10 μM

synthetic peptide encoded by exons I and II of the HEK gene (∈), 10 nM - 1 :M HEK I-VII (o), 10 nM - 1:M HEK IV-VII (x) or 1 nM -1 μM HEK I-III (9) were injected onto an sHEK-derivatised sensor chip. The residual responses are illustrated as percentage of the total response in the absence of competitor.

FIG. 3: Schematic comparison of native LERK7 with FLAG and Fc fusion constructs. In each case the precursor protein is depicted with an arrow leading to the final processed form. The original precursor protein is processed to remove the signal sequence and the hydrophobic glycophosphatidyl inositol linkage sequence (cleavage site indicated by arrow head) yielding the final GPI-linked form (FIG. 3A). LERK7-FLAG is engineered to stop before the hydrophobic tail and the native N-terminal signal sequence is replaced with the IL3 signal peptide and the FLAG epitope (FIG. 3B). FIG. 3C illustrates the LERK7-Fc construct where the hydrophobic tail of the native sequence is replaced by the Fc and hinge regions of human IgG_1. After processing this yields the disulphide linked homodimer (Bohme *et al.*, 1996, *supra*).

FIG. 4: Shows a comparison of the binding of LERK-Fc fusion proteins to sensor chip-immobilised HEK. Samples (50 μl) of purified fusion proteins comprising the human Fc domain and either LERK 1, 2, 3, 4, 5 and 7 at l0 μg/ml in BIAcore running buffer were applied onto an sHEK-derivatised sensor chip. The responses were recorded 20s after completion of the injection phase and are shown as relative BIAcore response units, (RU) compared to the response of an equal amount of human Fc domain used as a control in this experiment.

FIG. 5: Binding curves for the interaction of monovalent LERK-FLAG

fusion proteins with immobilised sHEK. Homogeneous preparations of CHO cell-derived LERK 3-FLAG (panel A) or LERK 7-FLAG (panel B) at increasing concentrations (8.22, 16.44, 32.88, 65.75, 131.5, 263, 526 nM) of LERK3-FLAG, and (1.25, 2.5, 5, 10, 20, 40, 80 nM) LERK7-FLAG were injected across an sHEK-derivatised sensor surface. BIAcore data for the association and dissociation phases were used to estimate corresponding kinetic rate constants on the basis of a one-to-one interaction model. (panel C). Free sHEK was estimated in samples with increasing sHEK and a constant LERK 7-FLAG concentration at equilibrium and used to calculate the equilibrium dissociation constant K_D by Scatchard analysis.

FIG. 6: Characterisation of bivalent ligand binding by generation of ternary sHEK/LERK-FLAG/anti-FLAG M2 mAb complexes *in situ*. Solutions (5 μg/ml) of purified LERK 7-FLAG (panels A, B) or LERK 3-FLAG (panels C, D) with (sensorgrams e,f), or without addition (sensorgram c) of cross-linking anti-FLAG M2 mAb (5 μg/ml) were injected across an sHEK-derivatised sensor surface (1) followed by a subsequent injection (2) of buffer (sensorgrams c, e) , M2 Mab (5 μg /ml, sensorgram d) or FLAG peptide (25 μg/ml, sensorgram f). For comparison, injections of buffer (l) followed (2) by M2 mAb or FLAG peptide (sensorgrams a and b, respectively) were performed in parallel experiments.

~~FIG. 7: Induction of HEK phosphorylation in LK63 cells by LERK 7-FLAG. A Western blot membrane probed with PY20 anti-phosphotyrosine antibody (panel A) was stripped and analysed with rabbit anti-HEK antibody (panel B). Lane l - FLAG-LERK 7/M2 antibody complexes, lane 2 - FLAG-LERK 3/M2 antibody complexes, lane 3 - FLAG-LERK 7, lane 4 -~~

FLAG-LERK-3 and lane 5-control medium alone.

FIG. 8: Effect of ectopic expression of the human HEK receptor extracellular domain and of soluble LERK-7 on the development of zebrafish embryos. Zebrafish embryos were injected with 10 pg of either HEK I-VII mRNA or LERK-7 mRNA and 5 pg marker mRNA during the first two cleavage divisions and raised at 28°C. Embryos are photographed from a lateral perspective in a-c, with dorsal to the right and from a dorsal perspective in d-f, with anterior up in each frame.

FIG. 8A: A non-injected zebrafish embryo at 12 hours post fertilization (hpf) showing normally developed otic vesicle, forebrain (open arrow head) and tail-butt (closed arrow head) and revealing a normal dorsal height from the yolk surface (H).

FIG. 8B: A zebrafish embryo at 12 hpf after microinjection with 10 ng HEK I-VII mRNA displaying strong developmental defects in the mid- and hind brain, poorly developed otic vesicle (open arrow head) reduced height of the dorsal axis from the yolk surface (H) and absence of somitic grooves and tail-butt (closed arrow head).

FIG. 8C: A zebrafish embryo at 12 hpf after microinjection with 10 ng LERK-7 mRNA displaying an identical phenotype to the HEK I-VII mRNA injected specimen.

FIG. 8D: Dorsal view of the non-injected embryo illustrated in a), revealing well-developed somitic grooves "lining up in register" along the midline.

FIGS. 8E & 8F: Dorsal views of embryos shown in (FIGS. 8B, 8C) respectively showing poorly developed somites.

FIG. 9: Analysis of HEK and LERK-7-mediated developmental effects by *in situ* hybridisation. Embryos were injected with

~~10 pg of either sHEK I-VII mRNA or sLERK-7 mRNA, allowed to develop for 12 to 13 hpf and fixed for *in situ* hybridisation with *pax-b*, *hlx-1*, *krox20 and myoD* digoxigenin-labeled riboprobes.~~

~~Embryos are photographed from a dorsal perspective with anterior to the top and posterior to the bottom of each frame; a, c, e, antero-dorsal view; b, d, f, posterio-dorsal view: (a, b) uninjected embryo at 12 hpf showing normal expression of *hlx*-1 the ventral forebrain, *pax-b* in the midbrain, *krox-20* in rhombomeres 3 and 5 of the hindbrain and *myoD* in the paraxial mesoderm; (c, d) HEK I-VII mRNA (10 pg) injected embryo at 12 hpf showing *pax-b* and *krox 20* expressing cells in the mid and hindbrain extensively displaced from the midline; *myoD* expressing cells of the paraxial mesoderm displaced from the midline are visible posterior to the brain; an intact *hlx-1* stripe is present anteriorly; (e, f) LERK-7 mRNA (10 pg) injected embryo at 12 hpf demonstrating a phenotype which is almost identical to that of the HEK I-VII mRNA injected embryo shown in (c, d).~~

FIG. ~~10~~7: ~~Ectopic expression of HEK IV-VII, missing exons I-III of the ligand-binding domain, does not affect the development of zebra-fish embryos;~~

~~FIG. 10A~~ Detection of expressed HEK I-VII and HEK IV-VII (far right lanes) in zebrafish embryos by Western blot and BIAcore analysis. Samples of lysis buffer or zebrafish lysates (10 embryos/0.1 ml) containing 25, 20, 5, 2.5 ng of HEK I-VII and HEK IV-VII were immunoprecipitated with anti-FLAG M2 mAb-agarose and analysed by Western blot. Zebrafish embryos injected with HEK I-VII mRNA or HEK IV-VII mRNA were lysed after 5h or 10 h and analysed in parallel lanes of the gel. Specific detection of FLAG epitope-containing HEK proteins corresponding to the expected molecular weight in embryos at 5 and 10 hpf demonstrates that the recombinant

fusion proteins were present in embryos throughout the period of development analysed here. Parallel BIAcore analysis of whole embryo lysates with sensorchips derivatised with native conformation-specific anti-HEK mAb (Boyd *et al.*, 1992, *supra*; Lackmann *et al.,* 1996) detects the intact HEK exodomain at an apparent concentration of 120 to 240 ng per embryo (5 to 10 hpf, respectively, data not shown).

~~FIG. 10B: A zebrafish embryo at 12 hpf after microinjection with 10 ng HEK I-VII mRNA displaying a strong example of the syndrome such as severe anterior defects, a reduced height of the dorsal axis from the yolk surface and absence of somitic grooves are visible.~~

~~FIG. 10C: 15 hpf embryo after injection with 10 pg HEK IV-VII mRNA. The morphology of these embryos is indistinguishable from that of non-injected control embryos (FIG. 8A).~~

~~FIG. 10D: The same embryo viewed under epi-fluorescence illumination to detect translation of coinjected E-GFP marker mRNA demonstrating widespread and high level expression.~~

~~FIG. 10E: Uninjected embryo at 12 hpf showing normal expression of *hlx-1* in the ventral forebrain, *pax-b* in the midbrain, *krox-20* in rhombomeres 3 and 5 of the hindbrain and *myoD* in the paraxial mesoderm.~~

~~FIG. 10F: HEK I-VII mRNA (10 pg) injected embryo at 12 hpf showing *pax-b* and *krox-20* expressing cells in the mid and hindbrain extensively displaced from the midline; *myoD* expressing cells of the paraxial mesoderm displaced from the midline are visible posterior to the brain.; an intact *hlx-1* stripe is present anteriorly.~~

~~FIG. 10G: HEK IV-VII mRNA (10 pg) injected embryo at 12 hpf demonstrating normal expression of *hlx-1, pax-b , krox20*~~

~~and *myoD*; by comparison with (e) the embryo in (g) is rotated approximately 30° anteriorly.~~

FIG. ~~11~~8: Dose response and phenotypic rescue of soluble HEK and LERK7-induced developmental defects. Batches of embryos, injected with indicated amounts of HEK mRNA (□), LERK7 mRNA (9) or a combination of 10 pg HEK mRNA and 100 LERK7 mRNA (□) and a constant amount of E-GFP mRNA (5 pg) were allowed to grow for 12-13 hpf before fixation and hybridisation with *pax-b*, *hix-1*, *krox20* and *myoD* DIG-labeled riboprobes. Embryos were analysed under a dissecting microscope and scored for aberrant patterns of gene expression. Non-injected control embryos were scored after an identical developmental period and identical handling to the injected embryos to control for defects due to the genetic background of particular parents in our strain. None of these embryos showed any reproducible or significant defects.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

We have now shown that monovalent ligand constructs interact with Hek with markedly different affinities, with LERK7/AL-1 being 50-fold more avid than LERK3. This suggests that these receptors do not show true redundancy, but rather use varying affinity for different, and possibly overlapping, ligand gradients for fine control of cell movement within the developing organism.

In this specification we describe studies of the exon structure of the HEK gene, particularly with regard to the exons encoding the extracellular region or exodomain of HEK and related RTKs to demonstrate a consensus structure for all these genes. Deletion mutants of the HEK exodomain were constructed based on these data and expressed protein obtained. With the elucidation of the HEK gene structure as a starting point, we exploited the specific interaction between

HEK and LERK7 in a biosensor-based strategy to identify the N-terminal, exon III-encoded cysteine-rich subdomain as a crucial part of the ligand binding domain.

These receptor exodomain constructs were analysed functionally in a dominant-negative approach by micro-injecting mRNA encoding either the full-length receptor exodomain (HEK I-VII) or the soluble ligands (LERK7), or a deletion construct of the receptor, in which the coding sequence for the ligand binding domain was absent (HEK IV-VII mRNA), into zebrafish embryos. Whereas injection of HEK I-VII mRNA and mRNA encoding soluble LERK7 had severe, dose-dependent effects on the development of the fish embryos. A phenotype comparable to non-injected control embryos was observed at moderate concentrations of injected HEK IV-VII mRNA and expression of comparable amounts of the endogenous protein.

In addition to severely impaired mid and hindbrain development, prominent effects on axis and somite formation were observed early during development (12-14 h post fertilisation), in accord with the early expression patterns of the putative zebrafish HEK homologue *rtk2* (N. Holder, personal communication) starting at 80-90% epiboly (9 hpf) as reported previously by Xu *et al.*, 1994). The defects are consistent with a failure of lateral cells to move towards the midline as part of the cell movements enacted during gastrulation. Furthermore, our results raise the possibility of an endogenous signal mediated by a LERK 7 homolog and required by lateral cells for migration towards the midline.

Our data demonstrate a pivotal role of HEK */rtk2* during early vertebrate embryogenesis, and indicate that a defined ligand/receptor interaction has critical functions at progressive developmental stages.

EXPERIMENTAL

MATERIALS AND METHODS

1. *Isolation and mapping of HEK genomic clones*

The HEK cDNA probes used to screen the human genomic

libary were PCR fragments amplified from plasmids containing full length HEK cDNA. The primers used were:

probe A (spans bases 74 to 116 as described by Wicks *et al.*, 1992, *supra*)

GTAGGAATTCCTCTCACTGCCCTCTGC (SEQ ID NO:9) and

GTAGGGATCCGGCCTCCTGTTCCAG (SEQ ID NO:10);

probe B (bases 1053 to 1124)

GTAGGAATTCCATGG CTTGTACCCGAC (SEQ ID NO:11) and

GTAGGGATCCCATAATGCTTGCTTCTC (SEQ ID NO:12);

probe C (bases 2 to 186)

ATGG ATGGTAACTTCTCCAG (SEQ ID NO:13) and

TCATTGGAAGGCTGCGGAAT (SEQ ID NO:14), and probe D (bases 909 to 1404)

GTAGTCTAGACAAGCTTGTCGACCAGGTTT (SEQ ID NO:15) and

GTAGTCTAGATCAAGCCTGATTAGTTG TGATGC (SEQ ID NO:16).

The mouse genomic library was screened with a MEK4 fragment isolated from a plasmid subcloned with MEK4 cDNA. The cDNA fragment spans bases 582 to 899 of the MEK4 sequence (Sajjadi *et al.*, 1991, New Biologist **3** 769-778) .

The genomic libraries used were human in λ FIX II vector, (Stratagene Cloning Systems, La Jolla) and mouse in λ FIX II vector (Stratagene) and λ DASH II vector (kindly provided by F.Kontgen, Walter and Eliza Hall Institute for Medical Research, Melbourne). Approximately 10^6 plaques from each library were plated, replica nylon membrane filters (Bio-Rad Laboratories, New York) were prehybridized at 42°C in 50% formamide, l0X Denhardt's solution, 0.05 M Tris-Cl pH 7.5, 1.0 M NaC!, 2.24 mM tetra-sodium pyrophosphate, 1% SDS, 10% dextran sulfate and 0.1 mg/ml sheared, heat-denatured herring sperm DNA, and the filters hybridized at 42°C for 16 hours. Washes were performed at 68°C in 0.1 x SSC, 0.1% SDS for I hour and in 0.1 x SSC, 0.5% SDS for a further 30 minutes. Positive clones were identified by autoradiography, purified by

subsequent screenings and isolated using standard methodology (Sambrook *et al.*, 1989, *supra*).

Exon-intron boundaries were determined by a combination of direct DNA sequencing, PCR, restriction analyses, and Southern blotting. Direct DNA sequencing of the genomic lambda phages and subcloned plasmid was performed using the ABI 373 DNA sequencer (Applied Biosystems, Melbourne, Australia). Sequencing and PCR primers used to characterize the HEK gene from human genomic clones were based on the HEK cDNA sequence (Wicks *et al.*, 1992, *supra*).

The exons found within the mouse genomic clones were amplified by PCR using degenerate primers specific to Eph family RTKs:- GTAGGCATGCAAGGAGA C(AC)TT(CT)AACC (SEQ ID NO:17) and CC (AG) ATGGGNACCAGCCA (CT) TC (SEQ ID NO:18).

The PCR products were then directly sequenced as described above using the degenerate primers.

2. *Production of HEK in CHO cells*

Soluble HEK and N-terminally FLAG-tagged HEK were prepared from transfected Chinese Hamster ovary (CHO) cell supernatants as previously described (Lackman *et al.*, 1996, Proc. Natl. Acad. Sci. (USA) **93** 2523-7). Deletion mutants of HEK were prepared by PCR using oligos based on the exon boundaries. HEK III and HEK IV were constructed using a 5' oligonucleotide based on the N terminal sequencee of the mature protein (Boyd *et al.*, 1992, *supra*) with a 5' XbaI-site GTAGTCTAGAGAACTGATTCCGCAGCCTTCCAA (SEQ ID NO:19) and 3' oligonucleotides based on sequences spanning exon IV GTAGTCTAGATCATGGAGGTCGGGTACAAGC (SEQ ID NO:20) and exon III GTAGTCTAGATCAAGCTTGGCACATAAAACCTC (SEQ ID NO:21)

respectively, followed by a stop codon and an XbaI site. To construct HEK IV-VII, a 5' oligo designed to span the 5' end of exon IV with a 5' XbaI site GTAGTCTAGACAAGCTTGTCGACCAGGTTTC (SEQ ID NO:22) and a 3' oligonucleotide based on the C-terminus of the exodomain with a stop codon and flanking XbaI site GTAGTCTAGATCATTGGCTACTTTCACC

AGAG (SEQ ID NO:23).

In each case the PCR products were cloned into the IL3 signal-FLAG-pEFBOS vector as previously described (Lackmann, 1996, *supra*). DNA was electroporated into CHO cells (Lackmann, 1996, *supra*), and high producer clones were selected by "dot blot" screening of culture supernatants on PVDF membranes, and the expected size of the recombinant proteins confirmed by SDS-PAGE and Western blot analysis using M2 anti-FLAG mAb and rabbit anti-mouse alkaline phosphatase (AP)-tagged mAb for detection by enhanced chemiluminescence (ECL, Amersham).

Deletion mutants were purified on M2 anti-FLAG affinity columns and elute with FLAG peptide according to the manufacturers instructions. Homogeneous preparations (> 95% by SDS-PAGE and silver staining) were obtained by anion-exchange chromatography (Mono Q, 5x50 mm, Pharmacia, Uppsala, Sweden) and size exclusion chromatography (Superose 12, 10 x 300 mm, Pharmacia, Uppsala, Sweden). The identity and concentration of the purified HEK proteins in the final preparations were confirmed by N-terminal amino acid sequence analysis and amino acid analysis and, where applicable, their native conformation confirmed on the BIAcore as previously described (Lackmann, 1996, *supra*).

3. *Production of LERK-3 and LERK7 (AL-1) expression constructs*

The 5' LERK7 oligonucleotide GTAGTCTAGACAGGACCCGGGCTCCAAGGC (SEQ ID NO:24) was based on the N-terminal amino acid sequence QDPGSKA (SEQ ID NO:25) of the mature protein, with a 5' tag sequence and XbaI site preceding the coding nucleotides. The PCR reaction was performed using an aliquot of a placental cDNA library (kindly provided by Dr Tracy Wilson, Walter & Eliza Hall Institute) and Taq EXTEND (Boehringer-Mannheim). A 490 bp product was detected on a 1.4% TAE/agarose gel. This was excised and the DNA purified using Geneclean II (BI0101). The PCR

product and the IL3 sig-FLAG-pEFBOS vector (Nicola *et al.*, 1996) were digested with Xbal and the vector treated with calf intestinal alkaline phosphatase to prevent re-ligation. After ligation correctly oriented clones were detected and verified by automated DNA sequencing as already described.

4. *Transfection of cells with LERK 3 and LERK 7 DNA*

Purified LERK 7-pEFBOS DNA was transfected into CHO cells. Briefly, 2 x 10^7 cells were suspended in 500-:l of PBS and 10 :g of LERK-pEFBOS DNA and 1 :g of pSV2neo DNA added. After mixing and transfer to a 0.4 cm electroporation cuvette (BioRad), the cells were electroporated at 270 V and 960 :F and the cells centrifuged through an FCS underlayer. Transfected clones were selected in medium containing 600 :g/rnl of G418. Individual clones were isolated and samples (5 :l) of CHO cell supernatants from confluent cultures were dotted onto a nitrocellulose membrane, air-died and re-hydrated in blocking buffer (5% skim milk powder/0.1% Tween 20 in PBS) prior to incubation with M2 anti-FLAG antibody at 1:1000 dilution. After washing the blot was incubated with horse radish peroxidase-conjugated rabbit anti-mouse Ig antibody (1:1000, Dako) in blocking buffer and following further washes developed with the ECL detection system (Amersham). Positives clones, indicated by signals above background, were retested by SDS-PAGE (12%) and Western blots prepared and probed as described above to confirm the presence of FLAG-tagged proteins of the expected molecular size.

5. *Production of FLAG-tagged LERK 7 /AL-1*

LERK7/AL-1 containing an N-terminal FLAG peptide was purified from transfected CHO cell supernatants by affinity purification on anti-FLAG mAb-conjugated agarose according to the manufacturer's protocol (IBI Kodak, New Haven CT) followed by MonoQ (Pharmacia Biotech) ion exchange chromatography in 20 mM Triis, pH 8.5/ 0.02% Tween 20 at 1 ml/min using a linear, 40 min gradient from 0-600 mM NaCl and size exclusion-HPLC (Superose 12, 10/30, Pharmacia Biotech) in 20

mM Tris. The homogeneity, concentration and identity of the purified proteins were confirmed by reverse phase-HPLC, SDS-PAGE, amino acid analysis and N-terminal amino acid sequence analysis as described (Lackmann *et al.*, 1996, *supra*; Simpson *et al.*, 1986).

6. *Synthesis of HEK-derived peptides*

A peptide having an amino acid sequence corresponding to residues Glu 21 to Gly 51 of the sequence shown in FIG. 1 was assembled by solid-phase peptide synthesis according to standard protocols, purified by reverse phase-HPLC and its mass confirmed by mass spectrometry

7. *Analysis of the interaction between HEK constructs and LERK 7*

The binding of various HEK constructs and derived peptides was analysed on the BIAcore optical biosensor (Pharmacia Biosensor, Sweden) using purified soluble HEK (corresponding to the entire extracellular domain) or LERK 7-FLAG derivatised CM 5 sensor chips and the interaction kinetics determined. The immobilization of HEK onto the sensor chip surface was performed essentially as described by Lackmann et al, 1996. LERK7-FLAG (47 :g/ml in 20 mM sodium acetate, pH 4.5) was coupled at 5 :l/min onto N-hydroxysuccinimide (NHS, 0.05 M)/N-hydroxysuccinimide-N-ethyl-N'-(diethylaminopropyl)-carbodiimide(EDC, 0.2 M) activated sensor chips (45 :l, 2 :l/min) to yield an increase in the response level of 2500 - 3000 response units (RU).

The interaction kinetics of LERK-binding to immobilised HEK was analysed from raw data of the BIAcore sensorgrams suitable for analysis using linear and non-linear kinetic models included in the BIAevaluation software (Biosensor, P., 1995, BIAtechnology Handbook, Pharamacia Biosensor AB, Uppsala, Sweden). All results recorded in this report were within the typical dynamic ranges of BIAcore measurements and the BIAevaluation software. Single component kinetics was derived from:

$$-k_d(t-t_0)$$

dissociation : $R = R_0.e^{-k_s(t-t_0)}$ (equation 1)

dissociation : $R = R_0/k_s.(1-e^{-k_s(t-t_0)})$ (equation 2)

or

: $R = R_{eq}(1-e^{-k_s(t-t_0)})$; $k_s = k_aC+k_d$ (equation 3)

where R_0 is the response time at time t_0, R_{eq} the steady state response level (not necessarily reached in the sensorgram) and C the molar concentration of the analyte. The two component dissociation was derived from:-

$$R = R_1 e^{(-k_{d1}.(t-t_0))} + (R_0-R_1) e^{-k_{d2}.(t-t_0)}$$

(equation 4).

Apparent affinities of LERKs 3, 4, 5, 7 were also derived from equilibrium responses according to:-

R SUB eq/C ~ = ~ K SUB A R SUB max -K SUB A R SUB eq (equation 5)

where R_{eq} and R_{max} are the equilibrium and maximum response levels, respectively. In addition to the analysis of ligand binding to sensor chip-immobilised HEK, the interaction between LERK-3 and LERK-7 with HEK was studied in solution. A constant ligand concentration was incubated with increasing concentrations of the soluble receptor. The free ligand concentration (F_{LERK}), estimated from the BIAcore™ response of a known LERK sample was used to calculate the concentration of bound receptor ($[B_{Hek}]$) or ligand ($[B_{LERK}]$), and free HEK (F_{Hek}) using the initial receptor concentration (T_{Hek}) and assuming in this case a single site interaction : $[F_{Hek}] = [T_{Hek}]-[B_{Hek}.B_{LERK}]$, where $[B_{Hek}.B_{LERK}] = [B_{Hek}]=[B_{LERK}]$.

Thus the dissociation constant, K_D, was estimated from:-

K SUB D ~ = ~ { [F SUB Hek].[F SUB LERK] } OVER { [B SUB Hek.B SUB LERK] } ~ = ~ [F SUB Hek] ~ { [F SUB LERK] } OVER { [B SUB LERK] } ~ (equation ~ 6)

according to Ward *et al.*, 1995, Biochemistry **34** 2901-7 and Scatchard

transformation yielded:-

{ [B SUB LERK] } OVER { [F SUB LERK] } ~ = ~ { 1 } OVER K SUB D ~ [F SUB Hek]

The effect of HEK-derived peptides on the interaction of HEK with LERK 7 was tested by incubation of a constant concentration of the ligand with increasing amounts of peptide prior to analysis on a HEK-derivatised sensor chip as previously described. The affinity surface was regenerated between subsequent injections of samples with a 35 :l-injection of 50 mM, 1,2-diethylamine/0.1% Triton X100, followed by two washes with-BIAcore.running buffer (HEPES- buffered saline/0.005% Tween 20).

8. *Fish care and embryo collection*

Wild type zebrafish were obtained from St. Kilda Aquarium (Melbourne) and were kept essentially as described by Westerfield *et al.*, 1995, The Zebrafish Book, 3rd ed., University of Oregon Press, Oregon. Embryos were obtained by natural spawning between a small number (4-10) of male and female fish. Embryos were removed from the spawning tanks within 20 minutes of fertilisation, cleaned in system water, and transferred to the injection apparatus.

9. *mRNA synthesis*

Constructs encoding HEK I-VII, HEK IV-VII and LERK7 were generated by PCR from the cDNA constructs described above. In each case the 5' oligonucleotide was based on the IL-3 signal sequence and the 3' oligonucleotides were as above except that Bgl II sites were used to clone the PCR products into the pSP64TK vector. mRNA from the HEK and LERK7 constructs and a control E-GFP CDNA construct were transcribed *in vitro* using the mMessage mMaker kit (Ambion, Texas) and resuspended in water at a concentration of 0.1 mg/mL in small aliquots. Integrity of the RNA was checked by denaturing gel electrophoresis of the resulting products. Immediately prior to injection, aliquots of HEK I-VII, HEK IV-VII or LERK7 were thawed and mixed with water and E-GFP

mRNA to a final concentration such that either l00 pg, l0 pg, or 1 pg of the mRNA, and 5 pg of the E-GFP mRNA were delivered to each embryo.

10. *Microinjection*

Approximately 600pl of mRNA, dissolved in water at the desired concentrations, was injected into one-, two- or four- cell embryos under a Wild stereo microscope using Leitz micromanipulators (Leitz, Wetzlar, Germany) and compressed nitrogen. The needle was positioned under the blastoderm in the region of cytoplasmic streaming. Successful injection was judged in the first instance by a visible bolus of fluid in the embryo. Uptake and translation of mRNA by the embryo was measured by including 5 pg mRNA encoding E-GFP as a marker in each injection. injection of over l00 pg E-GFP mRNA per embryo does not cause developmental defects. The translation of the injected HEK mRNA was measured at intervals during embryogenesis by Western blotting. Ten embryos per time point were lysed in embryo lysis buffer (25 mM Tris-HC1, pH 7.4, 0.5M NaCl, 1% Triton X-100), immunoprecipitated with anti-FLAG M2 mAb-conjugated agarose, subjected to SDS-PAGE, transferred to PVDF-Plus membranes and Western blotted with M2 mAb. Detection of antigen was visualised using ECL. Protein levels were quantitated by densitometric comparison with HEK-FLAG mass standards run in the same gel.

11. *Analysis of developmental defects*

The effects on embryonic growth of each of the injected mRNAs were measured in two ways. Firstly, embryos were allowed to grow for twelve to thirteen hours post-fertilisation (five to eight somite stage; Kimmel *et al.*, 1995) their gross morphology was noted under a dissecting microscope, and the perturbation of early gene expression patterns was assayed by *in situ* hybridisation using digoxigenin-labeled RNA probes. Secondly, embryos were scored as defective if a typical pattern of gene expression was aberrant e.g. misshapen, missing or ectopic.

EXAMPLE 1: ***Genomic Structure of the Extracellular Region of the HEK Gene***

A human genomic library was screened with the HEK cDNA probes described above, and positive clones were characterized by restriction mapping and Southern blotting using exon-specific oligonucleotides derived from the HEK cDNA sequence. Exons were identified by sequencing subcloned genomic fragments, or by directly sequencing the phage clones with HEK oligonucleotides. A clone containing exon II was not obtained, and its sequence was inferred from the 3' and 5' junctions of exon I and exon III respectively. Sequences for intron-exon splice junctions were matched using the "GTAG rule" (Mount, 1982), and the results, summarised in Table 1 and FIG. 2A show that the extracellular region consists of seven exons interrupted by six introns.

Exon boundaries were determined by sequencing nonoverlapping λ FIX II clones, and are shown for the extracellular domains of the HEK gene. This sequence is the flanking region of the start methionine, as deduced from the published cDNA sequence (Wicks *et al.*, 1992, *supra*).

The parallel isolation and analysis of clones from a mouse genomic library containing exons II and III of SEK I, exon III of BSK, and exon IV of ESK indicates that this arrangement is a general feature of genes encoding Eph family RTKs. The results are illustrated in FIG. 1, and together with reports on the structure of chicken CEK 5 gene (Connor & Pasquale, 1995, Oncogene **11** 2429-2438) and splice variants of other Eph family RTKs (Sajjadi et *al.*, 1991, *supra*; Maisonpierre *et al.*, 1993, Oncogen **8** 3277-3288) suggest that exon structure is highly conserved within the Eph family.

Exon I contains all of the 5' untranslated sequence and the first 88 bp of the coding sequence which includes the signal peptide, and together with exon II encodes the first 31 residues of the mature protein starting at residue 21 of the sequence shown in FIG. 1 (Wicks *et al.*, 1994,

Genomics **19** 38-41). Exon III contains 10 of the 20 conserved cysteine residues characteristic of the Eph family RTKs. Although previous reports have described this cysteine-rich domain of Eph family kinases as being immunoglobulin-like, we found no significant homology between exon III and any other protein domains in the database using the ALIGN sequence alignment program. It has been suggested that the carboxy terminus of exon III is similiar to an EGF-like repeat as it contains the consensus sequence motif CnCxCxxGYnC (Table 2, Connor & Pasquale, 1995, *supra*). Although the genomic organization is not typical of a repeat (see below), we believe that exon III arose from a fusion or shuffling of exons which once encoded an EGF-like module.

Database analysis of the exon IV sequence showed matches with EGF repeat regions in other proteins. Moreover, the six conserved cysteine residues follow a CnCnCnCXCnC pattern, which is characteristic of an EGF-like repeat (Table 2) found within the EGF precursor gene (Bell *et al.*, 1986, Nucleic Acids Res. **14** 8427-8446). EGF-like repeats are usually encoded by a discrete exon, as noted in the genomic organization of EGF precursor protein, LDL receptor, and human factor IX (Anson *et al.*, 1984, EMBO J. **3** 1053-1060; Sudhof *et al.*, 1985, Science **228** 815-822; Bell *et al.*, 1986, *supra*).

The exon-intron borders of the two fibronectin (FN) domains correlate exactly with the borders predicted by analysis of the amino acid sequence. The first fibronectin type III repeat (FN I) is encoded by a single exon, which also contains the remaining four conserved cysteine residues found within Eph family RTKs. Exon VI and exon VII encode the second fibronectin repeat (FN II). The genomic organization of the fibronectin type III repeats is typical of the type III homology units found within the fibronectin gene and other proteins containing fibronectin repeats (Oldberg and Ruoslahti, 1986, J. Biol. Chem. **261** 2113-216; Giger *et al.*, 1995, Eur. J. Biochem. **227** 617-628). The fibronectin type III units are encoded by either a single exon or by two exons; however, the units

which are spliced out in various protein isoforms are usually those encoded by a single exon (Oldberg & Ruoslahti, 1986, *supra*). This is also observed with transcripts of Eph family members which lack FN I (Maisonpierre *et al.*, 1993, *supra*).

EXAMPLE 2: ***Assignment of the Disulphide bonds in HEK***

The structural importance of disulphide bonds for the architecture of a protein or protein domain is undisputed. Although the connectivities of 8 of the conserved cysteines in exons III and IV are inferred from the respective domain structures, as shown in Table 2, assignment of remaining disulphide bridges in exon III and an experimental confirmation of the predicted Cys-Cys bonding pattern were critical requirements for a reliable expression of conformationally stable receptor subdomains.

In a strategy adapted from Hodder *et al.* ,1996, we analysed reduced and non-reduced tryptic maps of the minimally-glycosylated HEK by analytical RP-HPLC, to rapidly identify disulphide-containing fragments as absorbance peaks unique to the RP-HPLC profile of the non-reduced tryptic digest. Automated N-terminal amino acid sequence analysis and mass-spectrometry of these peptides enabled identification of the disulphides within the native HEK receptor exodomain.

Assigment of the following peptides was confirmed:-

Cys_{7I} - Cys_{189}	(CI-C4)
Cys_{257} or $_{259}$ - Cys_{270}	(C8/9-C10)
Cys_{306} - Cys_{322}	(CI5-CI6)
Cys_{362} - Cys_{365}	(CI7-CI8).

EXAMPLE 3: ***Expression and purification of HEK subdomains***

A series of HEK cDNA constructs, as illustrated in FIG. 2B were transiently transfected into COS 7 cells, and the resulting culture supernatants were screened by immunodetection with anti-FLAG M2 mAb for the production of recombinant proteins. Constructs yielding proteins of the expected size in culture supernatants were stably transfected into

CHO cells. Appreciable expression levels were found for HEK proteins, designated HEK I-VII (exons I-VII), HEK I-IV (exons I-IV), HEK I-III (exons I-III), HEK IV-VII (exons IV-VII), and HEK V-VII (exons V-VII). Western blot analysis of the expressed proteins revealed the expected apparent molecular sizes for HEK I-VII (68 kD), HEK I-IV (36 kD), HEK I-III (33 kD), HEK IV-VII (40 kD), and HEK V-VII (36 kD). Interestingly, no expression was observed for any of the protein constructs containing the exon III-encoded domain, but missing the first 31 amino acids of the mature HEK protein (encoded by exons I and II; amino acids 21-51 of the sequence shown in FIG. 1), suggesting impaired transcription, translation or stability of these constructs.

Purification of HEK I-VII, HEK I-IV, HEK I-III and HEK IV-VII by anti-FLAG mAb-affinity chromatography followed by anion exchange HPLC (HEK I-VII and HEK I-IV) or size exclusion-HPLC (HEK I-III and HEK IV-VII) yielded homogenous preparations, as shown in FIG. 2B, which were suitable for analysis of their interactions with LERK7 on the BIAcore.

EXAMPLE 4: ***Binding of various LERK-Fc fusion proteins to sensorchip-immobilized HEK***

Receptor-ligand interactions within the Eph family RTKs has mainly been studied by modified indirect Scatchard analysis of human IgG-Fc fusion proteins of ligands or receptors binding to receptor or ligand transfected cells, respectively (Beckmann *et al.*, 1994, *supra*; Davis *et al.*, 1994, *supra*; Ceretti *et al.*, 1995, *supra*; Bennett *et al.*, 1995). To evaluate if BIAcore analysis also detected the interaction of various LERKs with HEK, we compared the binding of bivalent, Fc-fusion proteins of LERK (LERKS 1 to 5) and LERK 7 (FIG. 2C) to sensor chip-immobilised HEK exodomain. Each ligand sample was injected at concentrations between 0.1-10 :g/ml (approximately 0.8 to 80 nM) across the sensor chip. A sample containing 10 :g/ml of the recombinant. Human Fc fragment was used as a control. The relative binding response units (RU) of various

samples at 10 :g/ml are illustrated in FIG. 4, indicating comparable responses for LERKs 3, 4, 5 and 7 which were considerably greater than the responses of LERK 1 and LERK 2. Apparent dissociation constants derived from equilibrium responses (equation 4) at the four highest concentrations suggested a decreasing order of nanomolar affinities, as follows:-

LERK 7 > LERK 3 > LERK 4 >> LERK 5 (data not shown).

The interactions with LERKs I and 2 did not reach equilibrium responses in our experiments, and hence precluded estimation of dissociation constants. Only background binding was seen with the control recombinant Fc construct, alone.

EXAMPLE 5: ***Interaction kinetics of LERK 3-FLAG and LERK 7-FLAG binding to HEK***

To evaluate the contribution of bivalency of Fc ligand constructs to the interaction kinetics, we performed binding experiments with monovalent forms of LERK 3 and LERK 7. Corresponding FLAG-tagged fusion proteins were expressed in CHO cells and purified to homogeneity from culture supernatants of selected clones by anti-FLAG mAb affinity chromatography and ion exchange HPLC. The identity of the recombinant ligand proteins was confirmed by N-terminal amino acid sequence analysis.

A qualitative comparison of the BIAcore data, illustrating binding of increasing amounts of LERK 3-FLAG, (FIG. 5A) and LERK 7-FLAG (FIG. 4B) to a HEK sensor chip, reveals marked differences in the kinetics of the two interactions. The LERK3/HEK interaction is characterized by extremely fast on and off rates, and comparable responses of LERK3 or LERK7 binding to HEK were found only at approximately 30-fold higher LERK3 concentrations in the applied sample.

Kinetic analysis of the association and dissociation phases using a single component model yielded apparent association and dissociation rate constants of k_a = 4.8 $\forall$ 0.13 x 10^5 $M^{-1}s^{-1}$ and k_d = 6.1 $\forall$

$0.8 \times 10^{-3}\ s^{-1}$ for LERK 7, $k_a = 3.7 \forall 0.9 \times 10^5\ M^{-1}s^{-1}$ and $k_d = 0.26 \forall 0.06\ s^{-1}$ for LERK 3. Apparant dissociation constants $K_D = 1.2 \times 10^{-8}$ M for LERK 7-FLAG and $K_D = 5.9 (\forall 0.4) \times 10^{-7}$ M for LERK 3-FLAG were estimated. Analysis of the raw data revealed good fits to linear, "one-to-one" interactions, yielding Chi square values of 0.64 ∀ 0.18 and 0.47 ∀ 0.06 for the LERK 7 and LERK 3 reactions, respectively. The apparent equilibrium affinity constant for LERK 7 was substantiated by Scatchard analysis of the in-solution, interaction (Ward *et al.*, 1995, *supra*), yielding an identical dissociation constant of $K_D = 1.\ 2 \times 10^{-8}$ M. On the other hand, the affinity of the LERK3/FLAG interaction was too low to obtain reliable data by "insolution" analysis.

Cross linking of LERK-FLAG with anti FLAG mAb alters interaction with HEK

We next addressed the possibility that the differences observed in the binding of either FLAG-tagged and Fc-tagged ligands to sensorchip-immobilised HEK were due to increased avidity of the divalent Fc tagged ligands. To quantitatively examine this effect *in situ,* we assembled bivalent ligand/mAb complexes before or during BIAcore experiments by cross-linking FLAG-tagged LERK 7 (FIGS. 6A, 6B) and LERK-3 (FIGS. 6C, 6D) with the anti-FLAGm M2 mAb.

The interactions of preformed LERK-FLAG/M2 mAb complexes (FIGS. 6B, 6D, graph e) with a HEK-derivatised sensor chip resulted in 3- to 5-fold increased BIAcore responses and markedly reduced off-rates of the ligand/antibody complexes compared to the non-complexed LERK-FLAG proteins (FIGS. 6A, 6C, graph c), reflecting the increased size, and indicating an altered avidity, of the interacting complexes. To confirm this, we injected FLAG peptide, to compete with the LERK-FLAG proteins for anti-FLAG mAb binding sites, into the dissociation phase of LERK-FLAG/M2 mAb complex (FIG. 6B, graph- f) . A dramatically increased of offf-rate in this experiment confirmed that the suggested increase in avidity was dependent upon anti-FLAG mAb-

mediated cross linking of LERK-FLAG.

Furthermore, injection of the anti-FLAG M2 mAb at the end of the first injection cycle resulted in a pronounced rise of the BIAcore signals, likely due to binding of newly-formed ligand/mAb complexes (FIGS. 6B, 6D, graph d). The increase of the responses above the levels observed with the monovalent ligands in the first part of the sensorgram, presumably reflects the increased size of the interacting ligand/mAb complexes. On the other hand, amplitude and slope of the response curve are also determined by the abundance and affinity of the ligand available for complex formation at the time of mAb injection. Since injection of equimolar amounts of LERK 3-Fc or LERK 7-Fc is expected to yield the same ligand concentrations at the end of the first injection cycles, differences in the amplitude of the response following mAb injection (compare graph d in FIGS. 6B, 6D) must portray primarily the different affinities of the LERK-FLAG/M2 mAb complexes. In support of this, the dissociation curves and the response levels of pre-formed (graph e) and *in situ* formed (graph d) ligand/mAb complexes at the end of the second injection cycle (after 1090 *s*) were found to be identical (FIG. 6D) or very similar (FIG. 6B).

Taken together, these strictly qualitative analyses demonstrate that anti-FLAG M2 mAb cross-linked LERK-FLAG dimers bind HEK with increased avidity due to decreased dissociation rates. The resulting response curves are qualitatively very similar to the sensorgrams of the corresponding LERK-Fc fusion proteins, suggesting that avidity plays a major role in the interaction kinetics of these ligand constructs.

EXAMPLE 6: ***Induction of HEK phosphorylation in ligand-treated cell cultures***

In addition to the kinetic analysis of the LERK-HEK interaction, we compared the ability of either LERK 3 or LERK 7 to mediate transphosphorylation of HEK in LK63 cells, which have been shown to express the receptor constitutively (Boyd *et al.*, 1992, *supra*).

LK63 cell cultures were incubated with buffer or solutions containing either LERK 3-FLAG, LERK 7-FLAG" or pre-formed complexes of these ligands with anti-FLAG M2 mAb. In the latter samples the concentrations of LERK-FLAG proteins and M2 mAb were adjusted to provide divalent ligand constructs by occupancy of both binding domains of the mAb with ligand-FLAG. The HEK receptor was then immunoprecipitated from the cells and analysed by western blot analysis. A Western blot membrane probed with PY20 anti-phosphotyrosine antibody was stripped and analysed with rabbit anti-HEK antibody to identify FLAG-LERK 7/M2 antibody complexes, FLAG-LERK 3/M2 antibody complexes, FLAG-LERK 7 and FLAG-LERK 3 compared to control medium alone. ~~The results in FIG. 7 illustrate the analysis with PY20 anti-phosphotyrosine mAb (panel A) followed by re-probing the stripped blots with rabbit anti-HEK antibody.~~ Phosphotyrosine analysis showed no significant differences between control ~~(lane 5)~~, LERK 3-FLAG ~~(lane 4)~~ or LERK 7-FLAG ~~(lane 3)~~ treated samples. In contrast, incubation of cells with LERK 3-FLAG/M2 mAb complex induced a small but significant increase ~~(lane 2)~~, and incubation with LERK 7-FLAG/M2 mAb complex ~~(lane 1)~~ gave a dramatic increase in phosphotyrosine content of HEK. Corresponding bands on the anti-HEK probed blots ~~(panel B)~~ show no significant difference in total HEK protein between the experimental groups.

EXAMPLE 7: ***BIAcore analysis reveals the exon III encoded Cys-rich region of the HEK exodomain as the LERK-7 binding domain***

The interactions between Eph family receptors and their ligands have commonly been analysed by an indirect Scatchard analysis of divalent receptor-exodomain/IgG_1 Fc fusion proteins binding to ligand-expressing cells, revealing equilibrium dissociation constants in the low nanomolar range (Winslow, 1995, Neuron **14** 973-981; Beckmann *et al.*, 1994, *supra*; Davis *et al.*, 1994, *supra*; Gale *et al.*, 1996, *supra*). In all these previous studies, the contribution of the avidity of the bivalent Fc-

fusion proteins has not been appreciated. In contrast, we have found an estimated K_D of 12 nM for the binding of LERK 7 to BIAcore sensor chip-immobilised monovalent HEK.

To evaluate the contribution of the various HEK subdomains to the receptor/ligand interaction, we followed a similar strategy and performed a kinetic BIAcore analysis on the binding of HEK I-VII, I-IV, I-III and HEK IV-VII to sensor chip-immobilised LERK 7. Deconvolution of the BIAcore raw data during the association and dissociation phases and Scatchard analysis of the equilibrium responses (Biosensor, 1995, *supra*) demonstrated monovalent, linear receptor/ligand interactions.

Substantially lower dissociation constants (i.e. higher affinities) of 18-29 nM, due to increased association rate constants (FIG. 2D), were observed for the interaction between LERK 7 and the HEK subdomain constructs HEK I-IV and HEK I-III (FIG. 2E). On the other hand, the very similar apparent dissociation constants of 72∀15 and 62∀12 nM for sHEK and FLAG-tagged HEK I respectively, and insiginificantly higher equilibrium dissociation constants as shown in FIG. 2E, suggested that an N-terminal addition of the FLAG epitope had no effect on the interaction between HEK and its ligand. Higher diffusion rates of the significantly smaller HEK subdomain constructs I-IV and I-III, and possibly an improved accessibility of the ligand binding interface, are the most-likely reasons for the apparently increased affinity of these constructs observed in the BIAcore experiments.

Importantly, no binding of HEK IV-VII to immobilised LERK 7 was observed at any of the concentrations tested (16-500 nM), thus identifying the ligand binding site as lying within the N-terminal portion encoded by exons I-III of HEK. To evaluate the contribution of the most N-terminal 31 amino acid residues of the mature HEK protein (amino acids 21-51 of FIG. 1), encoded by HEK exons I and II, we performed in-solution competition studies with a synthetic peptide, corresponding to this part of the HEK exodomain. The results, illustrated in FIG. 2F, suggest that the

presence of the 31-residue N-terminal peptide at concentrations up to 10 :M had no effect on the receptor/ligand interaction, whereas addition of HEK I-III or HEK I-VII resulted in a dose-dependent reduction of the BIAcore response. Taken together, these results unambiguously demonstrate that the cysteine-rich domain encoded by HEK exon III contains a crucial part of the ligand binding domain.

EXAMPLE 8: ***The effect of ectopic HEK expression in the developing zebrafish***

Analysis of Eph family RTKs and their ligands has centered largely on their role in axon guidance (reviewed in Maller *et al.*, 1996, *supra*; Friedman & O'Leary, 1996, *supra*; Tessier-Lavigne, 1995, *supra*), a process that occurs relatively late in embryogenesis. However, Eph family RTK are expressed at much earlier stages in embryogenesis (Cheng & Flanagan, 1994, *supra*; Henkemeyer, M., 1994, *supra*; Xu *et al.*, 1994, *supra*; Gilardi-Hebenstreit, 1992, Oncogene **7** 2499-2506; Nieto *et al.*, 1992, *supra*; Scales *et al.*, 1995, Oncogene **11** 1745-1752; Lickliter *et al.*, 1996, Proc. Natl. Acad. Sci. USA **93** 145-150), and little is known about what role they might play at this stage. To address this role we have used the zebrafish, a model which has been previously shown to be tractable to analysis of early embryonic events (Xu *et al.*, 1995, Development **121** 4005-4016; Xu *et al.*, 1996). These studies were remarkable because they demonstrated that ectopic expression of a mouse or Xenopus homologue of the zebrafish *rtk1* gene could be used to perturb development. Moreover, the effects were specific to the targeted gene in that defects were confined to regions of the zebrafish embryo that expressed endogenous *rtk1*.

The putative zebrafish HEK homologue, *rtk2*, is expressed during gastrulation from 80 to 90% epiboly in the dorsal axis and in a ring around the yolk plug. As epiboly completes, higher expression levels are seen in the anterior neuraxis and in lateral cells of the neural plate aligned approximately with the mes-met boundary (Xu *et al.*, 1994, *supra*). This

localisation is similar to the patterns of the MEK 4 transcript in the mouse embryo, which are seen at day 8.5 (10 somites) and show high levels of expression at day 9.5 in the mid and hindbrain and within the paraxial mesoderm of the somites (Cheng & Flanagan, 1994, *supra*).

The present inventors reasoned that ectopic HEK exodomain expression may cause defects in brain development and somite organisation. Also, a high-affinity, 1:1 interaction between the HEK exodomain and monovalent LERK7 has been characterised and the need for ligand crosslinking (~~FIG. 7~~ as hereinbefore described) or cell association (Wilson *et al.*, 1995) for RTK activation suggests that both, receptor exodomain and soluble ligand could serve as antagonists of signalling via the zebrafish HEK homolog.

The present inventors also reasoned that ectopic HEK exodomain expression may cause defects in the brain formation and somite of the zebrafish embryo. Therefore, the effect of a soluble, secreted form of the HEK RTK and of the ligand LERK 7 was tested on zebra-fish development. This was compared this with a mutant HEK lacking the exon III-encoded LERK-7 binding domain identified previously. A soluble form of the mouse HEK homolog, MEK 4, has been isolated from an embryonic cDNA library (Sajjadi *et al.*, 1991, *supra*), suggesting that the early mouse embryo is exposed to this form of the HEK protein. mRNA encoding HEK I-VII or encoding LERK7-FLAG (LERK 7 mRNA), was introduced into zebrafish embryos at the single, two and four cell stages by microinjection into the yolk cell immediately under the blastoderm. mRNA introduced at this stage becomes ubiquitously distributed throughout the embryo (~~FIG. 10D~~). We detected the presence of a FLAG epitope-containing protein corresponding to the expected molecular weight in embryos from 5 hpf until 10hpf (FIG. ~~10A~~7), at an apparent concentration of 0.5 - 1 ng/embryo, demonstrating that the protein was present in embryos throughout the period of development analysed here.

EXAMPLE 9: ***Injection of HEK exodomain mRNA causes patterning defects in early embryogenesis***

The effect of ectopic expression of the human HEK receptor extracellular domain and of soluble LERK 7 on the development of zebrafish embryos was determined. Zebrafish embryos were injected with 10 pg of either HEK I-VII mRNA or LERK 7 mRNA and 5 pg marker mRNA during the first two cleavage divisions and raised at 28°C.

A non-injected zebrafish embryo at 12 hours post fertilization (hpf) showed normally developed otic vesicle, forebrain and tail-butt and revealing a normal dorsal height from the yolk surface.

A zebrafish embryo at 12 hpf after microinjection with 10 ng HEK I-VII mRNA displayed strong developmental defects in the mid- and hind brain, poorly developed otic vesicle reduced height of the dorsal axis from the yolk surface and absence of somitic grooves and tail-butt.

A zebrafish embryo at 12 hpf after microinjection with 10 ng LERK 7 mRNA displayed an identical phenotype to the HEK I-VII mRNA injected specimen.

A dorsal view of the non-injected embryo described above, revealed well-developed somitic grooves "lining up in register" along the midline. However, dorsal views of injected embryos respectively showing poorly developed somites.

Animals injected with HEK mRNA or LERK7 mRNA developed a consistent syndrome in a concentration dependent manner. Inspection of the animals between 11 and 15 hpf revealed defects involving reduced dorsal axis height from the yolk cell, disorganised anterior neuraxis, and disorganised somite boundaries ~~(FIGS. 8A-F)~~.

In severe cases of the syndrome at 12 hpf ~~(FIGS. 8B and 8E)~~ there was little morphological differentiation visible along the anterior-posterior aspect of the dorsal axis. Axial tissue was flattened over the yolk cell and somites were elongated laterally, much as in the *trilobite* mutant (Hammershmodt *et al.*, 1996; Kane *et al.*, 1996; Solnica-Krezel *et al.*,

1996; Stemple *et al.*, 1996), and disorganised, often out of register across the dorsal midline. The anterior neuraxis was also disorganised so that optic vesicle formation was retarded, and the characteristic mid- and hindbrain seamentation visible at 13 hpf was reduced or absent ~~(FIGS. 8B, 8E, 8C, 8F)~~.

In less severely affected embryos, the defects were predominantly confined to the anterior portion of the dorsal axis.

EXAMPLE 10: ***Analysis of marker gene expression***

In order to better understand the nature of the defect and to allow a more objective quantitation of the proportion of embryos displaying defects, embryos injected with mRNA encoding soluble HEK and soluble LERK7 at three different mRNA concentrations (100 pg, l0 pg and 1 pg per embryo) were fixed between 12 and 13hpf, and marker gene expression was analysed. Animals were considered defective if in *situ* hybridisation with probes to *hlx-1* (Fjose, 1994), *paxb* (Krauss, 1991,), *krox20* (Oxtoby & Jowett, 1993), and *myoD* (Weinberg *et al.*, 1994) revealed abnormal patterns consistent with ectopic gene expression. A dose-dependent effect of HEK mRNA was seen across two orders of magnitude mRNA concentration (FIG. ~~1~~8). The nature of these defects is consistent with ~~the~~ gross morphological observations ~~(FIGS. 9A, 9B) presented above~~.

Embryos were injected with 10 pg of either sHEK I-VII mRNA or sLERK 7 mRNA, allowed to develop for 12 to 13 hpf and fixed for *in situ* hybridisation with *pax-b*, *hlx-1, krox20 and myoD* digoxigenin-labeled riboprobes.

Uninjected embryo at 12 hpf showing normal expression of *hlx*-1 the ventral forebrain, *pax-b* in the midbrain, *krox 20* in rhombomeres 3 and 5 of the hindbrain and *myoD* in the paraxial mesoderm. HEK I-VII mRNA (10 pg) injected embryo at 12 hpf showing *pax-b* and *krox 20* expressing cells in the mid and hindbrain extensively displaced from the midline. *myoD* expressing cells of the paraxial mesoderm displaced from

the midline were visible posterior to the brain. An intact *hlx-1* stripe was present anteriorly. LERK 7 mRNA (10 pg) injected embryo at 12 hpf demonstrated a phenotype which is almost identical to that of the HEK I-VII mRNA injected embryo.

The most profound defect compared with normal, uninjected embryos, is the failure of the mid, and hind brain and trunk paraxial mesoderm to fuse across the. dorsal midline ~~(FIGS. 9C-F)~~. The forebrain region was intact, as indicated by a single axially-located stripe of *hlx-1* expression in the ventral forebrain. Cells expressing *paxb* and *krox20* of the mid-and hindbrain respectiively are arrayed in lateral stripes at some distance from the dorsal midline. Non-injected control embryos, or embryos injected with with E-GFP mRNA alone, do not show this defect ~~(FIGS. 9A, 9B)~~. Thus a large gap separating left and right halves of the embryo is present from the posterior limit of the forebrain until the anterior level of the somites. This analysis suggests that there has been a failure of the cells of the mid-and hind-brain to converge to the dorsal axis correctly. Disorganised *myoD* expression confirms ~~(FIGS. 9D, 9F)~~ the observation in living embryos that many somites are out of register across the midline. This defect could result from a failure of lateral cells to converge to the midline, a conclusion consistent with the laterally-extended somitic segmentation seen in live embryos. However, a disruption of anterior-posterior patterning processes cannot be ruled out. A coherence of phenotypes in response to exogenous expression of either receptor exodomain or soluble ligand indicate speciffic rather than promiscuous activation of the endogenous HEK homologue by a putative zebrafish homologue of LERK 7.

We verified this notion by markedly reducing the number of defective embryos by co-injecting HEK mRNA together with LERK 7 mRNA ~~(not shown)~~. Importantly, our experiments confirm a strict conservation of structural and functional specificity of Eph family RTK in vertebrate development.

EXAMPLE 11: ***Deletion of the ligand binding domain of HEK rescues embryonic development***

The assigmnent *in vitro* of the domain in HEK that is required for high affinity binding to LERK 7 by methods as described in Example 4, was tested *in vivo* by introduction of mutated versions of soluble HEK into zebrafish embryos. We have shown that the extodomain of HEK requires the presence of the sequence encoded by exon III for high affinity binding to LERK 7. This assignment suggests that removal of exon III from the HEK mRNA injected into zebrafish embryos should abrogate developmental defects due to the interaction of HEK with ligands through the ligand-binding domain. Therefore, HEK !V-VII mRNA, was injected into zebrafish embryos at the same range of concentrations at which the full-length HEK mRNA. had produced defectiive development. The resulting embryos were assayed and scored for disrupted marker-gene expression patterns as described above.

A zebrafish embryo at 12 hpf after microinjection with 10 ng HEK I-VII mRNA displayed a strong example of the syndrome such as severe anterior defects, a reduced height of the dorsal axis from the yolk surface and absence of somitic grooves are visible.

A 15 hpf embryo after injection with 10 pg HEK IV-VII mRNA had a morphology indistinguishable from that of non-injected control embryos. The same embryo viewed under epi-fluorescence illumination to detect translation of coinjected E-GFP marker mRNA demonstrated widespread and high level expression.

Uninjected embryo at 12 hpf showed normal expression of *hlx-l* in the ventral forebrain, *pax-b* in the midbrain, *krox 20* in rhombomeres 3 and 5 of the hindbrain and *myoD* in the paraxial mesoderm.

HEK I-VII mRNA (10 pg) injected embryo at 12 hpf showed *pax-b* and *krox 20* expressing cells in the mid and hindbrain extensively displaced from the midline; *myoD* expressing cells of the paraxial

mesoderm displaced from. the midline are visible posterior to the brain.; an intact *hlx-I* stripe is present anteriorly.

A HEK IV-VII mRNA (10 pg) injected embryo at 12 hpf demonstrated normal expression of *hlx-1, pax-b , krox20* and *myoD.*

No significant developmental defects were detected in embryos injected with either I0 pg or I pg HEK IV-VII mRNA per embryo, either by gross morphological criteria ~~(FIG. 10C)~~, or by analysis of marker gene expression ~~(FIG. 10G)~~. Ubiquitous E-GFP expression ~~(FIG. 10D)~~ and detection of approximately 0.4 - 1.0 ng HEK protein per embryo by Western blotting (FIG. ~~10A~~7) during development indicate that the protein was widely and highly expressed. Thus the failure of cells to converge to the dorsal midline in embryos injected with HEK mRNA is a function of the exon III-encoded ligand binding domain, and is probably due to interaction of this domain with one or more LERK-like ligands present in the embryo. Interestingly, at high concentrations (100 pg per embryo) of injected HEK IV-VII mRNA, there was no difference in the proportion of defective embryos when compared to full length HEK mRNA-injected embryos. This indicates that at high concentrations of HEK exodomain, the developmental perturbation becomes effectively ligand independent and indicates a distinct receptor dimerisation interface.

DISCUSSION

Most of the studies of Eph family RTKs and their ligands carried out to date have been performed with divalent Fc fusion constructs of either ligand or receptor. The present inventors compared the binding of different LERK-Fc fusion proteins to HEK-derivatised sensor chips, and confirmed the suggested cross-reactivity of all the tested LERK-Fc constructs with HEK (FIG. 5). In accord with these reports, the interaction between HEK and Fc constructs of LERKs 1 and 2 was distinctively weaker than binding of LERKs 3 and 4, which in our experiments yielded similar BIAcore responses to LERK 7. On the other hand, while the previously published affinities of LERKs 1, 2 and LERK 5-Fc for HEK are

very similar (18, 43, and 23 nM, respectively; Beckmann *et al.*, 1994, *supra*; Cerretti *et al.*, 1995, *supra*), we could estimate apparent dissociation constants only from equilibrium responses of LERKs 3,4,5 and LERK7-Fc (K_Ds Of 5, 6, 24 and 3 nM, respectively) , whereas binding of LERKs 1 and 2 was too weak for a kinetic analysis. In addition, biphasic binding was reported previously only for the interaction between HEK-Fc with LERK 2, where a low affinity constant of 430 nM was found (Beckmann *et al.*, 1994, *supra*). Our comparative analysis of the association and dissociation phases of two candidate HEK ligands, LERK 3 (Kozlosky *et al.*, 1995, *supra*) and LERK 7 (Lackmann *et al.,* 1996, *supra*) indicated a concentration-dependent increase of the apparent dissociation rate constants (not shown), and an increasingly poor fit to the assumed one-component dissociation model.

A significant deviation of the divalent LERK-Fc kinetics from linear, single component interactions, suggesting a high-affinity interaction at low concentrations and a low-affinity interaction at high ligand concentrations confirms earlier studies by Hogg *et al.*, 1987, and Posner *et al.*, 1991, which demonstrate that kinetic models based on a one-to-one stoichiometry do not adequately describe the dissociation of bivalent solutes from surface-bound receptors.

The use of different approaches for the kinetic analysis of HEK/LERK-Fc interactions could explain the differences between the published data and the findings herein described. A direct evaluation of kinetic data from BIAcore progress curves is likely to be.more sensitive to changes in kinetic rate constants than indirect Scatchard analysis, which relies on the use of labeled mouse anti-human IgG antibodies to detect receptor-Fc fusion proteins bound to ligand-transfected cells (see, for example, Davis *et al.*, 1994, *supra*; Beckman *et al.*, 1994, *supra*). Competitive binding experiments of the LERK-Fc /HEK interaction in solution ~~(FIG. 7)~~ which are not affected by immobilisation artefacts and/or rebinding of dissociating ligand (Ward *et al.*, 1995, *supra*; Chatellier *et al.,*

1996) but rely on an "indirect" estimation of bound ligand or receptor (see Methods), gave no direct indication of biphasic kinetics from the slope of the Scatchard plots but yielded negative [B_{LERK}/F_{LERK}] values at low HEK concentrations, thus indicating artefactually high responses in these samples. The interaction of bivalent LERK 7-Fc containing only a single bound HEK, via the remaining free LERK 7 moiety to the HEK sensor surface, is a likely explanation for this artefact and confirms the concentration-dependent bivalency of the LERK-Fc/HEK interaction.

The comparative evaluation of all our binding data suggests that the bivalent, high-affinity interaction of two covalently linked binding domains of the LERK-Fc fusion protein with two adjacent, sensorchip-immobilised receptor molecules will compete at saturating ligand-Fc concentrations with a low-affinity, monovalent interaction of a single binding domain with a single receptor molecule. Similar effects have been described for the analysis of mAb/antigen interactions (see, for example, Chatellier *et al.*, 1995, *supra*) and for the interaction of dimeric IL-6 with the sensorchip-immobilised IL-6 receptor-exodomain (Ward *et al.*, 1996, *supra*).

In other studies Eph receptor/LERK interactions, the effect of solute bivalency has not been addressed. The necessity of ligand clustering for efficient receptor activation (Winslow *et al.*, 1995, *supra*; Davis *et al.*, 1994, *supra*; Cerretti *et al.*, 1995, *supra*) seemed to warrant the use of bivalent receptor ligand constructs. Such constructs were also used most recently in whole embryo *in situ* staining to confirm kinetic experiments performed with the same receptor-Fc constructs (Gale *et al.*, 1996, *supra*). On the other hand, it remains to be demonstrated that the interaction between membrane-bound ligands or receptor, and Fc-tethered bivalent receptors or ligands is a suitable system to study kinetics of physiological interactions of membrane-bound ligands and receptors (Pandey *et al.*, 1995, *supra*). Our experiments indicate that the artificial bivalency of the ligand constructs obscures an unambiguous analysis of

the reaction kinetics. In agreement with a report on the kinetics of the cell adhesion molecule CD2 and its GPI-anchored ligand, CD48 (van der Merwe *et al.*, 1993) we find that very low affinity, due to fast ligand dissociation, is apparently increased by high avidity-binding of multimeric ligand aggregates.

By analysing the binding of monovalent LERKs to HEK, either in solution (not shown) or using the (sensor) surface immobilised receptor (FIGS. 5 and 6), we were able to characterise the receptor/ligand interaction in detail. *In situ*-crosslinking of the monovalent ligands with a mAb during BIAcore experiments (FIG. 6) and prior to SE-HPLC analysis of LERK/HEK complexes (not shown) demonstrated qualitatively the effect of avidity on the interaction and confirmed the apparent higher affinities of bivalent ligand constructs. Differences in the dissociation phases of specific LERK interactions were concealed by the higher avidity of divalent binding components (FIG. 6) but have a major impact on the affinities of the monovalent ligands (FIGS. 5 and 6). Due to an extremely fast off-rate, the interaction of monovalent LERK3-FLAG with the immobilised receptor is very weak (FIG. 5B), an observation confirmed in solution which indicated an unstable, transient LERK3-FLAG/HEK complex. By contrast, binding of LERK 7-FLAG to HEK was characterised by a 40-times lower off rate and resulted in a stable receptor/ligand complex (not shown) which was confirmed by kinetic analysis of HEK binding to sensor chip-immobilised LERK 7, yielding an apparent K_D of 7.2×10^{-8} M. The dissociation rate of the LERK7-FLAG/HEK reaction was low enough to allow purification of the ligand/receptor complex from solution and to facilitate its characterisation by equilibrium sedimentation analysis.

The demonstration of a 1:1 stoichiometry confirms our results from BIA core and SE-HPLC experiments, indicating that HEK has a single binding site for LERK7, and explaining the necessity of ligand crosslinking for receptor activation and transphosphorylation demonstrated in this study ~~(FIG. 7)~~ and reported by others (Davis *et al.*, 1994, *supra*; Brambilla

et al., 1995, *supra*).

Our results clearly identify LERK7 as the best candidate for a physiological HEK ligand. Despite very similar apparent affinity constants for the LERK3 and LERK7 Fc fusion proteins, the interaction between their monovalent analogues and HEK differs substantially by a markedly higher dissociation rate of LERK3-FLAG protein. Cross-linking of the dissociating ligands with anti-FLAG-mAb decreases the dissociation rates and results in similar interaction kinetics for both ligands. Our results could suggest that the reported interactions between some of the LERKs and HEK are influenced by the choice of the ligand construct. Extrapolating our observations to the *in vivo* situation, it seems likely that LERK 3 functions as an effective ligand only at very high receptor and ligand densities on opposing cell membranes, whereas a stable LERK7/HEK complex persists at much lower receptor and ligand numbers.

SUMMARY

Identification of the ligand binding domain mechanism of perturbation of vertebrate embryogenesis with soluble HEK exodomain and soluble LERK 7

Specialised roles during vertebrate development have been described for a limited number of Eph-family RTK and their corresponding ligands (Cheng *et al.*, 1995, *supra*, Drescher *et al.*, 1995, *supra*, Nakamoto *et al.*, 1996, *supra*) and together with the high interspecies conservation of primary protein structures argues for conserved and specific functions of defined Eph receptor/ligand interactions. A comparison between the amino acid sequences for the extracellular domains of HEK and its murine (MEK4) and chicken (CEK4) homologues (96 and 91% overall identity, respectively) demonstrates highest identityin the exon III encoded domain (99.5 % and 99.3%, respectively; FIG. 2A) and suggests a high evolutionary constraint on the structure of this domain. By analysing the interactions of the receptor exodomain and

derived subdomains (FIG. 2) with its high-affinity ligand in *vitro* by BIAcore analysis and *in vivo* by expression in zebrafish, we were able to confirmthis notion. BIAcore experiments summarised in FIG. 2 demonstrate that the exon III-encoded domain is neccessary for high-affinity ligand binding.

Whereas expression of the HEK exodomain or of soluble LERK 7 during zebrafish embryogenesis results in dose-dependent disruption of midline development, expression of the truncated receptor lacking the ligand binding domain gives rise to unaffected embryos, emphasising the role of a HEK/LERK7 derived signal during vertebrate development. A partial rescue of the wildtype phenotype by co-injection of receptor exodomain and soluble LERK 7 mRNA suppports the specificity of the observed HEK-LERK 7 interaction.

We assume that the expressed soluble HEK protein acts in a dominant negative manner in the embryo. Previous studies in cell culture and in embryos have demonstrated that signalling through RTKs is inhibited by co-expression of kinase-deleted or truncated forms of the receptor (Honegger *et al.*, 1990; Frattali *et al.*, 1992a, b, Spritz *et al.*, 1992; Reith *et al.*, 1993; Peters *et al.*, 1994, Dumont *et al.*, 1994). This inhibition is thought to proceed via the formation of a dimeric complex on the surface of cells, in which an endogenous full-length length receptor pairs with the exogenous truncated receptor, resulting in a complex that cannot autophosphorylate, and hence is inactive in signaling (reviewed in Van der Geer *et al.*, 1994, *supra*).

The formation of these complexes can be either ligalnd dependent (Ueno *et al.*, 1993) or independent (Frattali *et al.*, 1992a,b, *supra*; LeviToledano, 1994). Expression of a kinase domain-deficient Eph family RTK has been used previously to disrupt the signaling of *rtkl/sekl* in zebrafish embryos (Xu *et al.*, 1996, *supra*).

We interpret the results described here using a model in which the secreted HEK extracellular domain binds soluble LERK7 or

binds to an endogenous receptor-ligand multimer and render this complex inactive. These soluble forms can also bind ligand (receptor) independently of the endogenous receptor (ligand), as demonstrated by our results in vitro thereby tiitrating the ligand (receptor) from the system. Both molecular mechanisms have the same effect: a decrease in the number of active cell surface receptor-ligand signaling complexes. Recent results indicate that transmembrane Eph receptor ligands may have the capacity to transduce a signal into the cells on which they are expressed (Holland *et al.*, 1996, Bruckner *et al.*, 1997). However, as LERK 7 is GPI-linked to the cell surface, ligation by HEK is not expected to generate a signal in this manner.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

TABLE 1

EXON	SPLICE ACCEPTOR	SPLICE DONOR
I* (88 bp)	M D C ccagcaac ATG GAT TGT	S N E TCC AAT GAA G gtaagcca
II# (65 bp)	V N L TC AAT CTA	S H G TCA CAT GGG
III (661 bp)	W E E ttcttcag TGG GAA GAG	M C Q ATG TGC CAA C gtaagagc

IV (156 bp)	A C R gtttgtag CT TGT CGA	A C T GCT TGT ACC C gtgagtag
V (336 bp)	R P P ctttgcag GA CCT CCA	N Q A AAT CAG GCT gtgagtac
VI (126 bp)	A P S ctttacag CT CCA TCA	Y E K TAT GAA AAG gtggggaa
VII (163 bp)	Q E Q cctcaaag CAG GAA CAA	S P D AGT CCA GAC T gtatgtat

TABLE 2 EGF-like repeats

C - (12) - C S C N A G Y - (6) - C	Hek	
C - (12) - C L C N A G H - (6) - C	SEK1	
C - (12) - C H C E P G Y - (8) - C	EPH	Exon III
C - (12) - C M C R P G Y - (8) - C	CEK5	
C - (12) - C T C K A G Y - (7) - C	ELK	
C - (13) - C A K C - (13) - C R C E N N Y - (11) - C	Hek	
C - (13) - C A K C - (13) - C T C D R G F - (11) - C	SEK1	
C - (13) - C L T C - (13) - C T C E S G H - (11) - C	EPH	Exon IV
C - (13) - C V H C - (13) - C V C R N G Y - (11) - C	CEK5	
C - (13) - C S H C - (13) - C T C R T G Y - (11) - C	ELK	
C - (7) - C S Q L C - (9) - C D C F P G Y - (8) - C	PREPRO-EGF	
C - (6) - C E H I C - (8) - C S C R E G F - (8) - C	PREPRO-EGF	
C - (6) - C S H V C - (8) - C L C P D G F - (7) - C	LDL Receptor	
C - (6) - C D Q F C - (8) - C S C A R G Y - (8) - C	Factor X	
C - (6) - C E Q F C - (9) - C S C T E G Y - (8) - C	Factor IX	
C - (6) - C A H Y C - (8) - C S C A P G Y - (8) - C	Protein C	
C (n) C (n) C (n) C x C xx G Y/F (n) C	CONSENSUS	

LEGENDS

TABLE 1

\# A 8 clone for this exon was not isolated; the exon boundaries are deduced from the boundaries of the adjacent exons.